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CELL SIGNALING AND REGULATION OF SMOOTH MUSCLE CONTRACTION FROM A PHYSIOLOGICAL AND A PATHOPHYSIOLOGICAL PERSPECTIVE

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Cell signaling and regulation of smooth muscle contraction from a physiological and a pathophysiological perspective

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my beloved family

ABSTRACT

The aim of this thesis was to examine the cell signaling and regulation of smooth muscle in different smooth muscle tissues and under pathophysiological conditions. The thesis is based on 4 papers and the experimental work is based on *in vitro* studies in mouse.

In **Paper I** we addressed the question if key characteristics of fast and slow smooth muscle types could be identified, based on contractile, cell signaling and metabolic properties. We examined 4 different smooth muscle mouse tissues (aorta, muscular arteries, intestine, urinary bladder) with a large span in contractile kinetics (V_{max}, maximal shortening velocity) based on SM-B expression (”fast” inserted myosin heavy chain). A quantitative PCR (qPCR) and Western blot approach was used to examine expression of key components in the contractile, metabolic and cell signaling pathways. A large variability between different smooth muscle tissues was found regarding contractile, cell signaling and metabolism. The reported main characteristics of fast and slow smooth muscle can serve as a basis for future studies of smooth muscle properties.

In **Paper II** we addressed the question if the two main Ca^{2+}-sensitizing pathways: RhoA-Rhokinase and protein kinase C (PKC) are altered in response to hypertrophic growth in the urinary bladder. We used a mouse model, partial urinary outlet obstruction, to induce hypertrophic growth of the smooth muscle. It mimics the over active bladder syndrome (OAB) in man, a pathophysiological condition affecting the urinary bladder with sudden and frequent urges to urinate, nocturia and urge incontinence. To examine if active force was altered, we used *in vitro* force recordings and direct nerve stimulation in open organ baths. Western blot analysis was used to determine if the relative protein expression of components mediating signaling in the RhoA-Rhokinase and PKC pathways. Direct nerve stimulation showed an increased cholinergic response in the hypertrophic smooth muscle, with a lower purinergic and increased nerve independent component. The hypertrophic smooth muscle also had an increased sensitivity to cholinergic stimulation and increased Rho dependent Ca^{2+} sensitivity that correlated with a lower phosphatase (MYPT1) expression and higher expression of both RhoGDI and RhoA. Based on these results and the profiling of the cell signaling in Paper I, it seems likely that hypertrophic growth of the urinary bladder induces transition from a fast smooth muscle type towards a slow smooth muscle type.

In **Paper III** we addressed the question if nonmuscle myosin (NMM) can be upregulated in response to hypertrophic growth in the urinary bladder and be involved in a PKC-induced contractile component observed in the hypertrophying urinary bladder. We examined the relative expression of NMM with Western blot and immunohistochemistry. Active force was analyzed using *in vitro* force recordings in open organ baths. In addition to smooth muscle myosin (SMM), the smooth muscle can express NMM, able to support a contraction with slow kinetics. However, in the urinary bladder NMM is only expressed during fetal life and downregulated shortly after birth. Western blot analysis showed an increased NMM expression in the hypertrophic smooth muscle compared to control. Immunohistochemistry showed an increased expression of NMM in the suburothelium, the smooth muscle layer and the serosa, for the hypertrophic urinary bladder compared to the control bladder. Direct

activation of protein kinase C (PKC) with PDBu gave a prominent contraction that was independent of Rhokinase. Blebbistatin is an inhibitor of nonmuscle myosin, with higher affinity for NMM than for SMM. The PKC induced contraction was almost completely abolished by blebbistatin, indicating that NMM is involved in this unique contractile component in hypertrophic urinary bladder. Smooth muscle from the hypertrophying urinary bladder can thus develop a unique PKC activated contractile component based on nonmuscle myosin, mainly localized to the serosa. However, this contractile component is not a major part of the normal muscarinic contraction, instead it may contribute to wall stiffness and be activated by other (unknown) upstream pathways.

In **Paper IV** we addressed the question if smooth muscle contraction is sensitive to metabolic inhibition and if there is a difference in sensitivity to metabolic block between fast and slow smooth muscle types, due to their different metabolic properties determined in Paper I. The mechanisms of metabolic control of smooth muscle are poorly understood. We approached this question by introducing a partial metabolic blocker (rotenone) of complex I in the mitochondria, resembling e.g. ischemic conditions due to atherosclerotic changes or ageing. To confirm that rotenone slows down the mitochondrial respiration, we measured oxygen consumption in the relaxed smooth muscle tissue and found about 50% inhibition by rotenone. We measured active force using *in vitro* force recordings in open organ bath, in the presence of blockers and activators to target membrane channels and cellular component that potentially might be affected by the metabolic stress induced by rotenone. Active force of the fast (urinary bladder) was more sensitive to rotenone than that of the slow smooth muscle (aorta), which correlates well with the metabolic profiling in Paper I. AMP-kinase, a metabolic sensor, is activated by metabolic stress (increased ADP:ATP and/or AMP:ATP ratios) and initiates a range of energy-saving processes in the cell. AICAR (AMPK activator) partially attenuated the rotenone effects on contraction, whereas dorsomorphin (AMPK blocker) dramatically increases the inhibitory effect of rotenone. Thus, AMPkinase appears to have a protective action during metabolic stress induced by rotenone in the smooth muscle.

In summary, this thesis demonstrates a large variability between fast and slow smooth muscle tissues regarding contractile properties, signaling and metabolism. Smooth muscle has an impressive ability to adapt during pathophysiological stress, e.g. in the urinary bladder. In the hypertrophic bladder muscle cell signaling is affected, increasing both the nerve induced cholinergic component and Rho-mediated Ca^{2+} sensitivity. In addition, hypertrophic smooth muscle can also develop a unique contractile component dependent on nonmuscle myosin that is activated by PKC. Partial metabolic block inhibits active force in the smooth muscle and can partially be prevented by AMPkinase. Compared to the slow smooth muscle, the fast smooth muscle is more sensitive to metabolic stress induced by rotenone.

LIST OF SCIENTIFIC PAPERS

I. **Boberg L**, Szekeres FLM, Arner A. Signaling and metabolic properties of fast and slow smooth muscle types from mice.
Pflugers Arch. 2018 470:681-691

II. **Boberg L**, Poljakovic M, Rahman A, Eccles R, Arner A. Role of Rho-kinase and protein kinase C during contraction of hypertrophic detrusor in mice with partial urinary bladder outlet obstruction.
BJU Int. 2012 109:132-40

III. **Boberg L**, Rahman A, Poljakovic M, Arner A. Protein kinase C activation of a blebbistatin sensitive contractile component in the wall of hypertrophying mouse urinary bladder.
Neurourol Urodyn. 2015 34:196-202

IV. **Boberg L** and Arner A. Metabolic regulation of contraction in fast and slow smooth muscle from mice.
Manuscript, 2018

CONTENTS

LIST OF ABBREVIATIONS

ACC2	acetyl-Coenzyme A carboxylase beta
ACh	acetylcholine
ADP	adenosine diphosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
BK_{Ca} channel	big conductance Ca^{2+}-activated K^{+} channel
CCh	carbachol
CPI17	small signaling protein, activated CPI17 is a very powerful inhibitor of the MLCP catalytic subunit, PP1c
DAG	diacylglycerol
FAS	fatty acid synthase
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
GTP	guanosine-5'-triphosphate
Gαq	isoform of G-protein coupled receptor, activates both the PKC and the Rho-Rho-kinase pathway
$G\alpha_{12/13}$	isoform of G-protein coupled receptor, activates the Rho-Rho-kinase pathway
G6P	glucose-6-phosphate, metabolite formed in the first step of glycolysis
G6PDH	glucose-6-phosphate dehydrogenase, enzyme in the pentose phosphate pathway
GLUT1	insulin-independent glucose transporter, facilitate transport of glucose over a plasma membrane independent of insulin
GLUT4	insulin-dependent glucose transporter, facilitate an insulin-regulated transport of glucose over a plasma membrane.
HEXO	hexokinase, regulatory enzyme in glycolysis
HPRT	hypoxanthine guanine phosphoribosyl transferase, housekeeping gene

HSL	hormone sensitive lipase
IP_3	inositol-trisphosphate
IP_3R	IP_3-receptor facilitates release of Ca^{2+} from SR
K_{ATP}	ATP sensitive potassium channel activated by low intracellular ATP concentration, and hyperpolarizes the plasma membrane.
LC_{20}	myosin regulatory light chain
LDH	lactate dehydrogenase, enzyme facilitating lactate formation
LPL	lipoprotein lipase
LC_{17a}, LC_{17b}	acidic and basic splice variant of myosin essential light chain
L-type Ca^{2+} channel	long-lasting (activating) voltage-gated calcium channel
LUTS	lower urinary tract symptoms
MLCK	myosin light chain kinase, phosphorylates and induces smooth muscle contraction
MLCP	myosin light chain phosphatase, dephosphorylates and induces smooth muscle relaxation
MCD	malonyl-CoA decarboxylase
MYPT1	myosin phosphatase regulatory targeting subunit, a subunit of MLCP
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMM	nonmuscle myosin
NO	nitric oxide
OAB	over active bladder syndrome
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate, a metabolite in glycolysis
PEPCK	phosphoenolpyruvate carboxykinase
PIP_2	phosphatidylinositol bisphosphat
PKA	cAMP-dependent kinase
PKC	protein kinase C
PKG	cGMP-dependent kinase
PLC	phospholipase C

PP1c	catalytic subunit of MLCP
PYRK	pyruvate kinase
qPCR	real-time polymerase chain reaction
ROCK	Rho-associated protein kinase.
RhoA	Ras homolog gene family member A, small GTPase protein
SK_{Ca} channel	small conductance Ca^{2+}-activated K^{+} channel
SM1	isoform smooth muscle heavy chain, unique sequence of 43 amino acids (~204 kDa) in C-terminal comprising a kinase phosphorylation site, SM2 is the shorter form
SM-B	"fast myosin", isoform of smooth muscle heavy chain with 9 amino acid insert in the N-terminal close to its ATPase site that creates a myosin construct that pushes the actin at a higher velocity. SM-A is the form lacking the insert.
SR	sarcoplasmic reticulum, an intracellular Ca^{2+} store of the smooth muscle
TFAM	mitochondrial transcription factor A

1 INTRODUCTION

1.1 THE CONTRACTILE MACHINERY OF SMOOTH MUSCLE

Smooth muscle is a heterogenic tissue that plays an important role in many elementary physiological processes in the body, i.e. in the vascular system, the airways, the gastrointestinal tract, the urinary bladder and the uterus, as presented in many textbooks of physiology (e.g. Boron and Boulpaep, 2005).

All muscle types generate force and shortening via actin-myosin interaction, a process regulated by variations in intracellular [Ca^{2+}] (Cooke, 1997). Myosin is a highly conserved ATP-dependent motor protein that is responsible for the actin-based motility. Smooth muscle contraction is due to a cyclic interaction of myosin cross-bridges with actin, in a similar manner as in the striated and cardiac muscles (Andersson and Arner, 2004). In this process, myosin converts chemical energy into mechanical work associated with the ATP hydrolysis reaction. This is a multistep enzymatic process where MgATP is hydrolyzed to MgADP and Phosphate (Pi). Smooth muscle contraction is dependent on constant energy flow from the cell metabolism, keeping the MgATP at high levels and removing MgADP and Pi. In comparison to striated muscles the smooth muscle ATP and cross-bridge turnover rates are 50-100 times slower, as reflected by a low ATPase and a low shortening velocity (Bárány, 1967), but a high economy while keeping force (e.g. Paul, 1980). The rate of cross-bridge dissociation during cycling is associated with ADP release and this enzymatic step is considerable slower in smooth compared to striated muscles (Löfgren et al., 2001), explaining the slow shortening velocity and high tension economy (i.e. maintenance of force related to ATPase). It should be noted that there is a large span in cross-bridge turnover between different smooth muscle types (Malmqvist and Arner, 1991), where the aorta and urinary bladder represent comparatively slow and fast smooth muscle types, respectively, as discussed below (section **1.5**).

1.2 ISOFORMS OF CONTRACTILE PROTEINS

The expression of the smooth muscle contractile proteins actin and myosin, involves different protein isoforms. Actin is a highly conserved protein that exhibits a considerable degree of homology between different isoforms. In smooth muscle four actin isoforms (α-, β-, and a smooth and nonmuscle variant of γ-actin) are expressed (cf. Drew and Murphy, 1997). Their functional characteristics are not yet fully understood, but the isoforms appear to have similar effects on cross-bridge turnover (Harris and Warsaw, 1993; Drew and Murphy, 1997). The actin isoform expression is thus not primarily responsible for the slow kinetics of smooth muscle or the difference between fast and slow smooth muscle types. Myosin in smooth muscle belongs to the filament forming myosin II superfamily (Walklate et al., 2016). The myosin molecule is a complex of six polypeptide chains arranged as a hexamer; containing

the two heavy chains (with the ATPase and actin binding sites), two regulatory light chains (20 kDa) and two essential light chains (17 kDa). Different isoforms of smooth muscle heavy chain are generated through alternative splicing in the COOH- (SM1 and SM2) and the NH2-terminal (SM-A and SM-B, cf. Andersson and Arner, 2004). The SM1 and SM2 have unique sequences in the filament forming region, comprising a kinase phosphorylation site in SM1. In the urinary bladder the ratio SM1/SM2 is lower in adult tissue, possibly suggesting differential role of these isoforms in development. However, the SM1 and SM2 isoforms do not have a major role in controlling the contractile kinetics (cf. Andersson and Arner, 2004). On the other hand, the isoforms in the NH2-terminal region (SM-A and SM-B) are close to the ATPase site of myosin. The SM-B myosin translocates actin at a higher velocity. Thus, the expression of the inserted SM-B smooth muscle myosin is considered a major regulator of smooth muscle kinetics (Kelley et al., 1993).

Also, the myosin essential light chains are expressed in two variants in smooth muscle through alternative splicing, the acidic (LC_{17a}) and the basic nonmuscle (LC_{17b}). Both a low shortening velocity and low ATPase activity are correlated with the LC_{17b} isoform (cf. Arner et al., 2003). It is thus most likely a combination between different isoforms of smooth muscle heavy chain (SM-A and SM-B) and smooth muscle essential light chains (LC_{17a} and LC_{17b}) that determines, or at least correlates strongly with, the contractile kinetics in different smooth muscle types, a fast smooth muscle has higher expression of SM-B and of LC_{17a}.

Besides the smooth muscle myosin II discussed above, filament forming nonmuscle myosin II can also be expressed in smooth muscle and support contraction (Morano et al., 2000). Two isoforms, encoded by separate genes, give rise to nonmuscle myosin heavy chain A (NM-MHC-A) and B (NM-MHC-B). NM-MHC-B is expressed in embryonic and newborn urinary bladders, and most likely critical for normal development. It can also be found in nonmuscle cells. Smooth muscles with high amounts of nonmuscle myosins have slow cross-bridge kinetics (Löfgren et al., 2003; Rhee et al., 2006).

1.3 ACTIVATION AND DEACTIVATION OF SMOOTH MUSCLE CONTRACTION

Activation of smooth muscle contraction is initiated by an increase in the cytosolic calcium level $[Ca^{2+}]_i$, either by Ca^{2+} influx from the extracellular space or by Ca^{2+} release from intracellular stores (i.e. sarcoplasmatic reticulum, SR).

Voltage gated L-type Ca^{2+} channels are a main source of Ca^{2+} influx and their activity are triggered by depolarization or receptor activation (Jaggar el al., 1998). Since they are dependent on membrane potential, K^+ channels have important roles in regulating the L-type Ca^{2+} channel opening probability. Opening of small conductance (SK) and large conductance (BK) K^+ channels, due to Ca^{2+} increase (Jaggar el al., 1998; Herrera and Nelson, 2002) or of ATP dependent K_{ATP} channels by low ATP (Tinker et al., 2014) will hyperpolarize the membrane and induce relaxation.

Free cytosolic Ca^{2+} binds to calmodulin and activates the Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK). MLCK is ubiquitously expressed in several muscle tissues and it phosphorylates the Ser-19 of the smooth myosin regulatory light chain (LC_{20}) and thereby allowing the cross-bridge cycle between myosin heads and actin to start (Horowitz et al., 1996; Somlyo and Somlyo, 2003). For the smooth muscle to relax, dephosphorylation of the LC_{20} by myosin light chain phosphatase (MLCP) is required. MLCP is a heterotrimeric protein that consists of a catalytic subunit (PP1c), myosin phosphatase regulatory targeting subunit (MYPT1) and a 20 kDa subunit of unidentified function (Somlyo and Somlyo, 2003; Andersson and Arner, 2004). MYPT1 affects the catalytic activity of MLCP and its activity is regulated by phosphorylation by several different kinases. In addition, the MLCP activity can be modulated by binding of CPI17; when phosphorylated, mainly by protein kinase C (PKC), it inhibits the phosphatase (Woodsome et al., 2001).

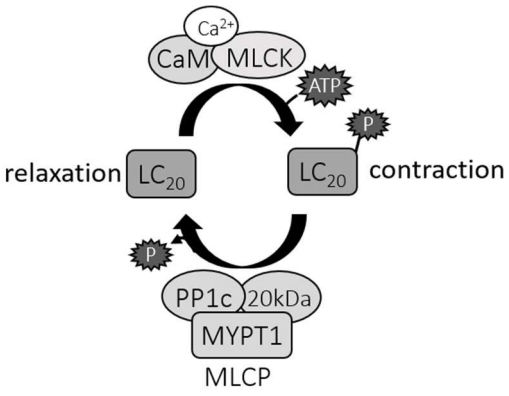

Figure 1. **Illustration of activation and deactivation of smooth muscle contraction.** Calcium (Ca^{2+}) binds to calmodulin (CaM) and activates myosin light chain kinase (MLCK), which phosphorylates the regulatory light chains (LC_{20}) and activates contraction. Smooth muscle relaxation requires dephosphorylation of the regulatory light chains by myosin light chain phosphatase (MLCP), consisting of three subunits PP1c, MYPT1 and a 20kDa subunit.

1.4 Ca^{2+}-SENSITIVITY MODULATION OF SMOOTH MUSCLE CONTRACTION

The main activation/deactivation pathway of smooth muscle is the Ca^{2+} activation of the MLCK and the dephosphorylation by the MLCP (section **1.3**, Figure 1). An important aspect of this regulation is that the "Ca^{2+}-sensitivity" can be regulated, i.e. at a given intracellular concentration of Ca^{2+} the amount of force developed can vary depending on the type of excitatory stimulus (cf. Rembold, 1992; Arner and Pfitzer, 1999; Somlyo and Somlyo, 2003). The Ca^{2+} sensitivity and extent of myosin light chain phosphorylation in smooth muscle are generally considered to be determined by the ratio between activated MLCK and activated MLCP. Agonist-induced activation of the smooth muscle can cause a shift in this balance in favor of the MLCK. Although MLCK activity can be modulated by cellular signaling, MLCP appears to be a key target for this Ca^{2+}-sensitivity modulation in smooth muscle. When MLCP is less active, dephosphorylation is inhibited and consequently the smooth muscle can be phosphorylated and contract at a lower $[Ca^{2+}]_i$ (Somlyo and Somlyo, 2003).

Protein kinase C (PKC) and Rho-kinase (ROCK) are the two main cellular pathways that terminate on the MLCP and inhibit its activity, which leads to an increased Ca^{2+} sensitivity of smooth muscle (Somlyo and Somlyo, 2003). These Ca^{2+}-sensitizing pathways are illustrated

schematically in Figure 2. The PKC and Rho-kinase pathways can be recruited either after ligand binding to G-protein coupled receptors in the plasma membrane or directly by an increase in intracellular [Ca^{2+}]. Force induced by the binding of various agonists to a G-protein coupled receptor (GPCR) is generally higher compared to force induced by depolarization (high K^{+}) of the plasma membrane, due to the Ca^{2+} sensitization associated with agonist binding to cellular receptors (Rembold, 1990). Depending on the GPCR isoform stimulated, different Ca^{2+}-sensitizing pathways can be recruited, i.e. most $G_{\alpha q}$ activate both the PKC and the Rho-Rho-kinase pathway whereas $G_{\alpha 12/13}$ mainly recruit the Rho-Rho-kinase pathway (Somlyo and Somlyo, 2003). In the resting smooth muscle cell, cytoplasmic RhoA-GDP is complexed with GDI (GDP dissociation inhibitor), thus promoting a deactivated Rho-pathway. Trimeric G-proteins are coupled to GEFs (guanine nucleotide exchange factors) and agonist binding to GPCR activates GEFs. GEFs activate the RhoA by catalyzing the exchange of nucleotide bound to RhoA, from GDP to GTP. Activated RhoA-GTP translocates to the plasma membrane and subsequently activates Rhokinase (ROCK). RhoA is returned to the inactivated GDP-bound from a process stimulated by GAP (GTPase-activating protein). ROCK is considered to phosphorylate a tyrosine residue on the myosin binding subunit (MYPT1) of MLCP and thereby inhibit phosphatase activity and increase the force development (Somlyo and Somlyo, 2003).

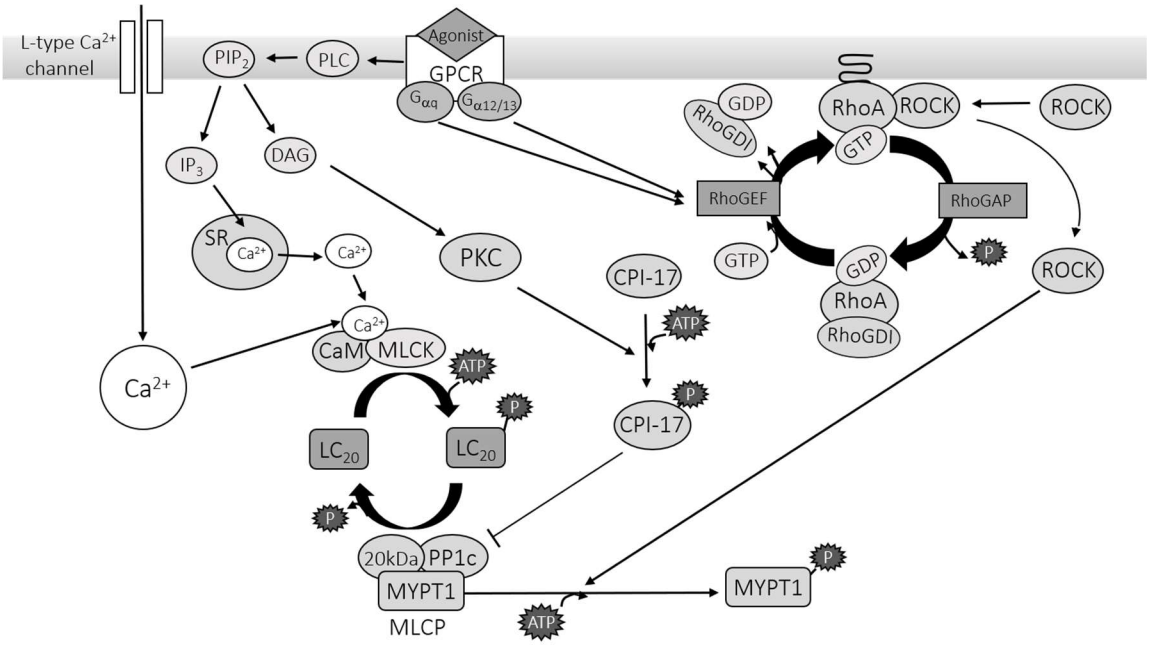

Figure 2. Main Ca^{2+} sensitizing pathways for activation of smooth muscle contraction. Depolarization of the plasma membrane opens L-type Ca^{2+} channels, leading to Ca^{2+} influx. Receptor activation can cause depolarization, but also generate inositol trisphosphate (IP_3) leading to sarcoplasmic reticulum (SR) Ca^{2+} release and to diacylglycerol (DAG) activation of protein kinase C (PKC). It also leads to activation of RhoA and Rhokinase (ROCK). Whereas Ca^{2+} activates the Myosin Light Chain Kinase (MLCK), both PKC (via CPI17) and ROCK inhibit the Myosin Light Chain Phosphatase (MLCP) and increase the Ca^{2+}-sensitivity.

Protein kinase C (PKC) can be activated by diacylglycerol (DAG) generated by phospholipase C and some isoforms also by Ca^{2+} (Morgan and Leinweber, 1998; Somlyo and Somlyo, 2003; Ringvold and Khalil, 2017). Receptor activation of phospholipase C (PLC) results in hydrolysis of PIP_2 (phosphatidylinositol-bisphosphate) into IP_3 (inositol-trisphosphate) and DAG (diacylglycerol). IP_3 binds to a receptor in the SR (IP_3R) which results in Ca^{2+} release. DAG activates protein kinase C (PKC) which in turn activates several pathways including its key substrate CPI17 via phosphorylation. Phosphorylated CPI17 is a powerful inhibitor of the MLCP catalytic subunit, PP1c (Woodsome et al., 2001).

In many ways, smooth muscle tone is modulated *in vivo* by both regulation of activation, as described above, and by activation of relaxation, e.g. in vascular and in erectile tissues. Nitric oxide (NO), released from endothelium, and specific receptor-stimulation are important relaxant mechanisms which directly, or via cGMP/PKG or cAMP/PKA, affect several pathways leading to lowering of intracellular Ca^{2+}-levels and the decreased Ca^{2+}-sensitivity (Morgado et al., 2012).

1.5 VARIABILITY BETWEEN DIFFERENT SMOOTH MUSCLE TISSUE TYPES

Smooth and striated (skeletal and cardiac) muscle is generally divided into different types according to their histological appearance (e.g. Boron and Boulpaep, 2005). The smooth muscle is however a heterogeneous group and displays a large variability in contractile, regulatory and electrophysiological characteristics. Different smooth muscle tissues also manifest great variation in their sensitivity to hormones, neurotransmitters, physical/chemical factors and pharmacological compounds. Agonist stimulation/binding can give different responses depending on the receptor expression and type of smooth muscle tissue, which allows for a remarkable adaptation of the muscle function to the physiological demands in the specific organ system.

Smooth muscle is regulated by the autonomic nervous system and exhibits a more diverse innervation and intercellular communication compared to skeletal and cardiac muscle. Emil Bozler (Bozler, 1948) proposed a model dividing smooth muscle into two discrete groups based on the innervation and electrical coupling: single unit (visceral) and multiunit. In the latter group, each smooth muscle cell receives input from neurons and there is little electrical coupling between the individual smooth muscle cells. Thus, the multiunit (e.g. in the ocular smooth muscle) allows better control/modification since smooth muscle cells can contract independently of each other. In contrast, single unit smooth muscle can contract coordinated as a unit, since it has extensive electrical coupling with only a few smooth muscle cells receiving neural input and can be spontaneously active (e.g. smooth muscle in the gut). This view can be found in many physiological textbooks. Nevertheless, Bozler´s model does not entirely explain the diversity of smooth muscle, since the innervation and electrical coupling of different smooth muscle tissues display a continuum of these characteristic rather than being discrete groups. The group of Somlyo and Somlyo proposed that smooth muscle should

be divided into “phasic” and “tonic” types (Somlyo et al., 1969), based on membrane properties and the contractile behavior exhibited after agonist activation. The phasic smooth muscles have a fast and transient force development compared to the tonic that exhibit a slow and sustained contraction. It has also been recognized that the “phasic” and “tonic” types differ in their excitation pathways, e.g. in the phosphatase activity, and several other parameters of the contractile system (Fisher, 2010).

It is interesting to note that smooth muscle exhibits a large span (5-7 fold) in contractile kinetics (as measured by the maximal shortening velocity), where slow smooth muscles (e.g. in the aorta) and fast smooth muscle (e.g. visceral smooth muscles) can be identified (Arner et al., 2003). The shortening velocity reflects the kinetics of the actin-myosin cross-bridge turnover, most likely the rate of ADP release, and has been correlated with the myosin isoform expression (section **1.1**). As discussed above, phasic (similar to fast) and tonic (similar to slow) smooth muscles differ in cellular signaling for contractile activation/deactivation (Fisher, 2010).

It seems also likely that the contractile turnover and the ADP sensitivity are correlated with properties in the metabolic pathways. An interesting approach for further characterization or classification of smooth muscle types could be to simultaneously consider the contractile, metabolic and signaling properties. This might enable a better understanding of the smooth muscle diversity, although such an approach has not been explored previously. It is well known that the smooth muscle has a low ATP turnover, and is thus generally considered an “economical” muscle. However, a more detailed analysis of the variability in energy metabolism between smooth muscle types is missing.

1.6 GENERAL FUNCTION OF KEY ENZYMES IN THE METABOLIC PATHWAYS

The constant energy flow from ingested nutrients containing proteins, carbohydrates and lipids is vital to uphold key processes in cellular function. The nutrients are metabolized in several steps to yield ATP (adenosine triphosphate), which is the universal chemical energy carrier in the cell. In the smooth muscle, the chemical energy is mainly transformed to mechanical work although activation/deactivation processes also are ATP dependent (e.g. Ca^{2+} translocation, myosin phosphorylation, ATP dependent membrane processes etc.).

Below a simplified overview of the general metabolic pathways involved in ATP generation in muscle are presented, focusing of key steps analyzed in **Paper I** of this thesis. A full account of all metabolic steps is not presented, but rather some key reactions used in the metabolic “profiling” of smooth muscle types. An overview is given below (cf. Alberts et al., 1989; Lehninger et al., 2000).

1.6.1 Glucose uptake and glycolysis

The mammalian plasma membrane contains a lipid bilayer that is impermeable to glucose, which thus requires specific membrane transporter to enter the cell. GLUT proteins, a family of integral membrane glycoproteins, facilitates transport of glucose across the plasma membrane. GLUT 1 and GLUT4 are expressed in (vascular) smooth muscle cells (Ebeling, 1998). *GLUT1* facilitates transport of glucose over a plasma membrane independent of insulin and assures a low level of basal glucose uptake required to sustain respiration in all cells (Ebeling, 1998).

GLUT4 is extensively expressed in adipose tissues and striated muscle. GLUT4 facilitate an insulin-regulated transport of glucose over the plasma membrane (Ebeling, 1998; Park, et al., 2005). To meet increased energy demand during muscle contraction, insulin induces translocation and fusing of GLUT4 vesicles to the plasma membrane, facilitating a rapid increase in the uptake of glucose (Park et al., 2005; Belman et al., 2014).

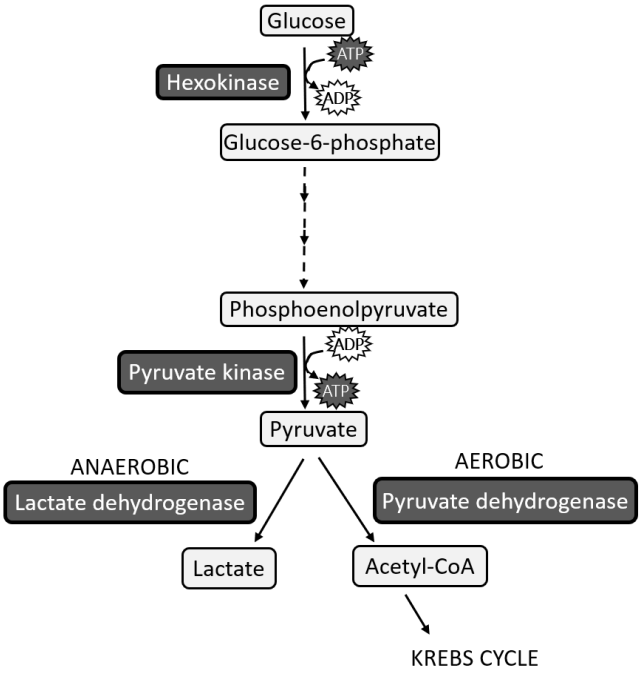

Figure 3. Simplified schematic of glycolysis. Glucose enters the cell and is broken down to pyruvate, via several irreversible and rate-limited steps. Hexokinase and pyruvate kinase controls the first and last of these steps, respectively. Depending on oxygen access and metabolic state of the cell, synthesized pyruvate can take different routes: aerobic as acetyl-CoA and enter Krebs cycle, or anaerobic and be converted to lactate by lactate dehydrogenase.

Glycolysis (**Fig. 3**) is a highly regulated and oxygen independent catabolic pathway that converts glucose into pyruvate via ten steps of enzyme-catalyzed reactions, generating ATP and NADH (reduced nicotinamide adenine dinucleotide). In the first step of glycolysis, *hexokinase* converts glucose to Glucose 6-phosphate (G6P) at the expense of one ATP, a rate-limited and irreversible step (Roberts and Miyamoto, 2015). The final step of glycolysis is also irreversible and rate-limited in the catabolic pathway, *pyruvate kinase (PYRK)* catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), resulting in the generation of ATP and pyruvate. Depending on the

metabolic state of the muscle and access to oxygen, pyruvate can either enter the Krebs cycle or be reduced to lactate, when aerobic or anaerobic condition is in favor respectively.

1.6.2 The lactate production

Krebs cycle is an oxygen-dependent pathway where ATP is generated from acetyl-CoA via a chain of enzyme-catalyzed reactions and the main source for ATP production in the cell. However, during anaerobic conditions *lactate dehydrogenase (LDH)* converts pyruvate and NADH to lactate and NAD^+ to meet the energy demand of the cell (**Fig. 3**). This enzymatic reaction is completely reversible and is regulated by feed-back inhibition, when lactate concentration is high. Interestingly, in the slow smooth muscle high amount of lactate is produced also during aerobic conditions (Paul, 1980). The underlying cause/function for this phenomenon has not been determined and different mechanisms have been proposed e.g. higher expression of hexokinase (Roberts and Miyamoto 2015) and glycolytic driven Na/K ATPase in the plasma membrane (Lynch and Paul 1983).

1.6.3 The gluconeogenesis

Gluconeogenesis (**Fig. 4**) is essentially a metabolic pathway (mainly in liver and kidney) converting non-carbohydrate carbon substrates (e.g. i.e. lactate, pyruvate, glycerol, fatty acids, glucogenic amino acids) to glucose, via a chain of enzyme-catalyzed reactions. Since glycolysis (breakdown of glucose, Fig. 3) involves several irreversible steps, gluconeogenesis needs to bypass these regulatory steps to produce glucose. *PEPCK (Phosphoenolpyruvate carboxykinase)* converts oxaloacetate to phosphoenolpyruvate (PEP) and carbon dioxide at the expense of one GTP. This first step of gluconeogenesis is strictly regulated and crucial for glucose homeostasis.

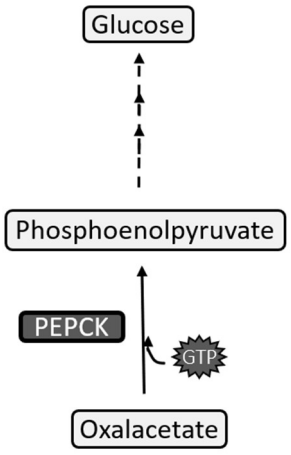

Figure 4. Simplified schematic of gluconeogenesis. To prevent the blood glucose levels from dropping too low, "new glucose" can be synthesized from non-carbohydrate carbon substrates via gluconeogenesis. This pathway is strictly regulated and involves the bypass of irreversible regulatory steps in glycolysis. PEPCK performs the first, thus crucial for glucose homeostasis.

1.6.4 The pentose phosphate pathway

The *pentose phosphate pathway* (PPP) is a metabolic pathway comprising an oxidative and a non-oxidative phase, where NADPH and 5-carbon sugars are generated, respectively (**Fig. 5**).

NADPH is important for reductive synthesis reactions within cells (e.g. fatty acid synthesis), and 5-carbon sugars are used in synthesis of nucleic acids and nucleotides. The first step in PPP is rate-limited by the enzyme *glucose-6-phosphate dehydrogenase (G6PDH)* that converts Glucose-6-phosphate (G6P) and $NADP^+$ into 6-phosphoglucolactone and NADPH (Chettimada, 2016). G6PDH is generally considered to be regulated by the ratio NADPH:$NADP^+$, thus as the ratio decrease the enzymatic activity is stimulated to generate more NADPH (Stanton, 2012).

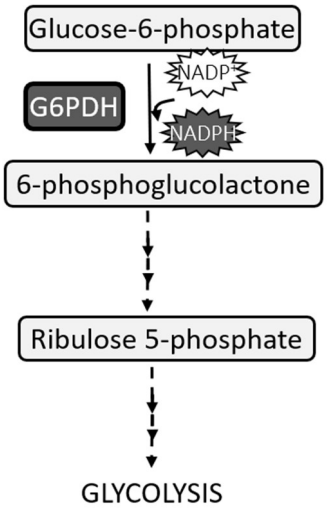

Figure 5. Simplified schematic of the Pentose Phosphate Pathway (PPP). The first step of PPP is rate-limited, controlled by Glucose-6-phosphate dehydrogenase (G6PDH) generating 6-phosphoglucolactone and NADPH from Glucose-6-phosphate and $NADP^+$. NADPH is important for the reductive synthesis reactions in the cell. 5-carbon sugars, also generated via PPP, are used in the synthesis of nucleic acids and nucleotides.

1.6.5 The mitochondrial turnover

Mitochondrial transcription factor A (TFAM) is crucial for embryonic development (Larsson et al., 1998), key activator of mitochondrial transcription and required for the replication of the mitochondrial genome (Kang and Hamasaki, 2005).

1.6.6 The metabolic sensor AMP-kinase

AMP-activated protein kinase (AMPK) is a key sensor of cellular energy status consisting of a heterotrimeric protein complex, with one catalytic subunit (α) and two regulatory subunits (β and γ) (Hardie and Carling, 1997). AMPK belongs to the family of serine/threonine kinases and is allosterically regulated by competitive binding between ATP and AMP or ADP to the gamma subunit, thus sensing the cellular energy status by monitoring the AMP:ATP and/or ADP:ATP ratios (Hardie and Carling, 1997). Metabolic stress (e.g. hypoxia, glucose deprivation, muscle contraction) results in increased cellular ADP:ATP and AMP:ATP ratios that activate AMPK. Activated AMPK initiates energy-saving processes, influencing/regulating a range of physiological and metabolic processes e.g. lipid metabolism, glucose metabolism, protein synthesis (Hardie and Carling, 1997; Hardie 2007 and 2011). Dysregulation of this AMPK has been implicated in different pathophysiological processes e.g. diabetes (Jeon, 2016).

1.6.7 The lipid synthesis and hydrolysis

To generate free fatty acids, *hormone sensitive lipase (HSL)* hydrolyzes intracellular triglycerides in stored adipocytes, whereas *lipoprotein lipase (LPL)* hydrolyze extracellular triglycerides.

HSL is regulated by the binding of various hormones (e.g. insulin, catecholamines) to specific receptors in the plasma membrane. Depending on the type of G-protein coupled to the receptor (G_s or G_i), adenylyl cyclase (AC) can be activated or inhibited. Activated AC increase the levels of cyclic AMP and activates PKA (protein kinase A, a cyclic AMP dependent protein kinase) that phosphorylate and activates HSL (Carey, 1998).

Lipoprotein lipase (LPL) is a multifunctional protein that is mainly present in adipose, skeletal and heart muscle tissue. LPL is localized to the luminal side of blood capillaries and generates free fatty acids by hydrolyzing extracellular triglycerides (TG) carried in circulating lipoproteins (chylomicrons and very low density lipoproteins).

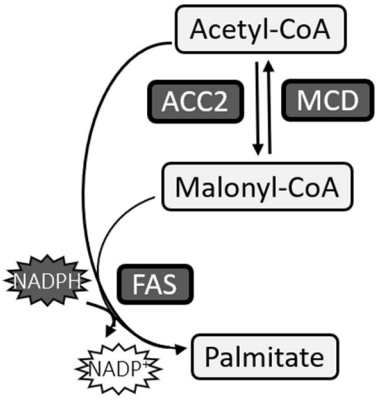

Figure 6. Simplified schematic of fatty acid metabolism. Fatty acid synthesis is dependent on the convention of Acetyl-CoA to Malonyl-CoA by ACC2. Next, FAS catalyze the convention of acetyl-CoA, malonyl-CoA and NADPH to palmitate, a precursor for long fatty acids. The intracellular level of Malonyl-CoA is tightly regulated, and MCD catalyze its formation back to Acetyl-CoA.

Fatty acid metabolism (**Fig. 6**) is tightly regulated and very receptive to physiological needs. Also, the fatty acid synthesis and degradation are reciprocally regulated and cannot be active simultaneously (Lehninger et al., 2000).

Acetyl-coenzyme A carboxylase beta (ACC2), catalyzes the formation of malonyl-CoA from acetyl-CoA and an irreversible step a rate-limiting step in biosynthesis of fatty acid. ACC2 is regulated by different hormones, resulting in the activation (by e.g. insulin) or inhibition (by e.g. glucagon, epinephrine) of enzyme activity (Lehninger et al., 2000).

Malonyl-CoA is an important metabolite in fatty acid biosynthesis, where a decreased cellular level result in increased fatty acid oxidation (Folmes and Lopaschuk, 2007). It is thus, tightly regulated and *Malonyl-CoA decarboxylase (MCD)* catalyzes the conversion of malonyl-CoA into acetyl-CoA and CO_2 (Foster, 2012).

Fatty acid synthase (FAS) is an essential enzyme involved in fatty acid synthesis, catalyzing the complex conversion of acetyl-CoA, malonyl-CoA and NADPH to palmitate (Jayakumar, 1995). Palmitate serve as a precursor for the synthesis of other long-chain fatty acids.

1.7 ADAPTIVE CHANGES IN SMOOTH MUSCLE CONTRACTION

Smooth muscle from different tissues exhibits a large span in contractile and signaling properties, and most likely also in characteristics of energy metabolism. An individual smooth muscle tissue can also adapt *in vivo* to altered functional demands and change to faster or slower contractile phenotypes with altered contractile protein expression (Arner et al., 2003). Changes in smooth muscle contractile activation/deactivation have also been reported for different patho/physiological conditions. For instance, altered RhoA-Rhokinase function has been described and several examples can be found, e.g. hypertension (Loirand and Pacaud, 2010), asthma (Chiba et al., 2010), and gastrointestinal dysfunction (Rattan et al., 2010). Changes in PKC have been found e.g. in bladder dysfunction (Hypolite and Malykhina, 2015) and vascular disorders (Ringvold and Kahlil, 2017). Altered Ca^{2+} sensitivity occurs in some conditions, e.g. in newborn bladders associated with altered MYPT expression (Ekman et al., 2005). Metabolic changes have been described but are rarely correlated with contractile or signaling properties. Although individual pathways can be affected in patho/physiological conditions and in some cases constitute a basis for pharmacological therapy (e.g. Fasudil®, a Rhokinase inhibitor used for treatment of vasospasm Feng et al., 2016; and other vascular conditions Shi and Wei, 2013), it would be important to relate any changes to contractile function and if possible obtain a more complete view on affected pathways. An identification of contractile, signaling and metabolic markers could be important for understanding pathological changes and for developing novel pharmacological therapies.

1.8 URINARY BLADDER AND INCONTINENCE

The physiological function of the urinary bladder is to store and expel urine. The smooth muscle (detrusor) in the wall of the bladder supports the three-dimensional structure of the organ and determines its wall tension. During the filling phase the detrusor muscle relaxes, allowing continuous expansion of the urinary bladder. Emptying of the urinary bladder requires a coordinated detrusor contraction and urethral relaxation (Andersson and Arner, 2004).

Impaired smooth muscle function is implicated in several pathophysiological conditions of the urinary bladder. Lower urinary tract symptoms (LUTS) is a major problem that increases markedly with age, affecting both male and females, especially in the elderly population (McDonnell and Brider, 2017) and can involve both overactivity and underactivity of the

detrusor muscle. Effective treatment of LUTS is missing and better knowledge of smooth muscle regulation during physiology and pathophysiology is necessary.

A common pathophysiological condition affecting the urinary bladder in humans as a part of LUTS is the overactive bladder (OAB) syndrome. OAB includes sudden and frequent urges to urinate, nocturia and incontinence. People living with OAB often experience embarrassment due to these factors, thus limit their social life or become isolated. Several treatments targeting receptors and signaling pathways have been introduced and proposed for OAB treatment although the success rate currently is low (Andersson, 2016). There are several conditions that can contribute to manifestation of OAB (Camões et al., 2015), e.g. ageing, ischemia and distension. An enlarged prostate can obstruct the urethra and induce hypertrophic growth of the urinary bladder, resulting in impaired bladder emptying and/or altered bladder storage of urine. The role of ageing and ischemia in OAB development is less understood although recent evidence suggest pelvic ischemia can be an important pathophysiological factor for detrusor overactivity and the overactive bladder syndrome (Andersson et al., 2017).

The urinary bladder dysfunction in OAB is multifactorial and likely involves both neurogenic and myogenic factors. Changes in detrusor properties, receptor function and cell signaling have been reported but the exact causes are not completely understood. Many animal models have been developed to study different parts of the pathophysiological mechanisms affecting the detrusor muscle in OAB. Partial urinary bladder outlet obstruction is an animal model that induces smooth muscle hypertrophic growth and hyperplasia, and thus mimics some features of the OAB symptomology in human (e.g. Malmgren et al., 1987). Although extensive work on different signaling pathways in OAB have be presented, the Rho and PKC pathways in relation to receptor induced contractile responses are not fully understood.

2 AIM

The general aim of this thesis project was to examine the cell signaling and regulation of smooth muscle in different smooth muscle tissues and under pathophysiological conditions. As pointed out in section **1.5**, smooth muscle exhibits a large variability in its contractile and signaling properties, although clear distinction in muscle groups is not available.

- The aim of **Paper I** was therefore to examine a range of smooth muscle tissues focusing on contractile, cell signaling and metabolic properties to examine if characteristics of fast and slow smooth muscle could be identified.

Cell signaling in smooth muscle is affected in several disorders (section **1.7** and **1.8**).

- The aim of **Paper II** was to examine a mouse model for urinary bladder hypertrophy in response to urinary outlet obstruction (mimicking OAB in man) and examine possible changes in the two main Ca^{2+}-sensitizing pathways, RhoA-Rhokinase and protein kinase C (PKC).

In experiments for Paper II we made an observation that a prominent sustained contraction could be elicited by direct PKC activation in the obstructed hypertrophying urinary bladder smooth muscle.

- The aim of **Paper III** was to examine the PKC-induced a contractile component in hypertrophying urinary bladder, with a special focus on the contribution of nonmuscle myosin.

In Paper I we examined a range of signaling and metabolic components in different smooth muscles. The effects of metabolic pathways on cell signaling and contraction in fast and slow smooth muscles are largely unknown.

- The aim of **Paper IV** is to examine the control of smooth muscle contraction by partial metabolic inhibition in a fast and slow smooth muscle type.

3 MATERIAL AND METHODS

3.1 ANIMALS AND OPERATING PROCEDURES

All studies in **Paper I-IV** were performed on adult C57/Bl6 mice (Taconic A/S, Denmark or B&K Universal AB, Sweden). Female mice were used in all studies; one exception is the qPCR in **Paper I** where male mice were used instead. The mice were housed at room temperature (12h light/12h dark cycle) and food and water were provided *ad libitum*.

The surgical procedure, partial urinary outlet obstruction (**Paper II, III**), was performed on adult female mice (10-12 weeks old) anesthetized with isoflurane. The urethra was identified via a lower abdominal midline incision, and a 0.5 mm metal rod was placed alongside the urethra and a 4-0 ligature was tied to create a partial intravesical obstruction. The metal rod was then removed, and the abdomen was sutured in separate layers. Immediately after the surgery, the mice were given a local injection of local anesthetic Bupivacain (Marcain®) in the wound area. The animals were also given Buprenorfin (Temgesic®) twice daily for two days. Sham-operated animals were used as controls, and these were subjected to the same operating procedures, except for that the ligature was left untied around urethra instead.

On daily basis, the general wellbeing of the operated animals was monitored, and no change in animal behavior or weight gain was noted. At 14-18 days after the surgical procedure, the animals were euthanized by cervical dislocation and the urinary bladder was removed weighed and used for *in vitro* experiments (**Fig. 7**). All husbandry and experiments were approved by the local animal ethics committee and conformed to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Council of Europe No 123, Strasbourg 1985.

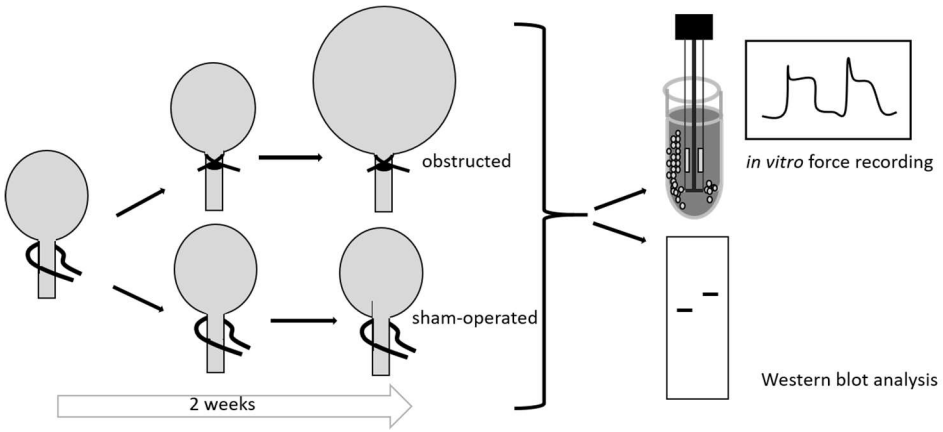

Figure 7. Illustration of hypertrophic growth in the urinary bladder. A ligature was tied around urethra, creating a partial intravesical obstruction, leading to hypertrophic growth in the urinary bladder. About 2 weeks after the surgery the animals were euthanized, and the urinary bladder removed for *in vitro* force recording or Western blot analysis.

3.2 ISOLATION AND PREPARATION OF TISSUES

In general, the animals were euthanized by cervical dislocation and smooth muscle tissues (aorta (**Paper I, IV**), mesenteric and femoral arteries (**Paper I**), ileum (**Paper I**), urinary bladder (**Paper I-IV**)) were quickly excised, transferred to ice-cold Krebs-Ringer solution (composition in mM: NaCl 123, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 20, $MgCl_2$ 1.2, glucose 5.5 and $CaCl_2$ 2.5) and dissected from the surrounding connective tissue and fat. The urinary bladder for the studies in **Paper II, III** was denuded of urothelium and circular muscle strips were cut out from the midline equator of the bladder. The samples were used for *in vitro* force recordings (Section **3.3**) for examination of pharmacological responses, nerve induced responses and determination of Ca^{2+} sensitivity and for determination of oxygen consumption (**3.4**). Samples were also chemically permeabilized /skinned (**3.5**) to examine Ca^{2+} sensitivity and fixed for immunohistochemistry (**3.8**). Tissues that were not used directly were rapidly frozen in liquid nitrogen in separate Eppendorf tubes and kept at -80ºC until further analysis with Western blot (**3.7**) or qPCR (**3.6**).

3.3 ISOMETRIC FORCE RECORDINGS ON INTACT MUSCLE PREPARATIONS

3.3.1 Mounting for isometric force recording (Paper II, III, IV)

For the *in vitro* force recordings in **Papers II, III, IV** circular muscle strips from the urinary bladder were cut out from the midline equator. In each end of the muscle strip (about 7 mm long and 0.3 mm thick) a 6-0 ligature was tied to enable mounting of the tissue in open organ baths (in 50 ml glass, Papers II and III or 5 ml Myograph System 610M, DMT, Paper IV). The aorta in **Paper III** (approximately 3-4 mm segment length length), was mounted on two thin metal rods in open organ bath (Myograph System 610M, DMT). After mounting the muscle strips in the open organ baths, they were stretched to optimal length (passive tension to about 5 mN and 4 mN, for aorta and bladder, respectively, cf. Davis et al., 2012) and allowed to equilibrate for 45 min in a Krebs-Ringer solution (bicarbonate buffered physiological salt solution at 37ºC, see composition in previous section) gassed with 95%O_2/5%CO_2 (pH 7.4). For each sample 2-3 contractures induced by high K^+ (80 mM) were induced to confirm reproducibility of contractions and determine an initial force response used for normalization of subsequent force responses.

Several agonists and antagonists were applied in the *in vitro* experiments. **Table 1** summarizes the main substances, their action and in which paper they were used.

3.3.2 Sensitivity to carbachol and αβ-methylene ATP (Paper II)

The cholinergic and purinergic dose-force relationships were determined in detrusor muscle from obstructed urinary bladders (**Paper II**). First, muscle preparations were activated with increasing concentrations of carbachol (a cholinergic agonist, 100 nM to 100 μM). For each carbachol induced contraction the initial peak and sustained force after 5 min were determined. Subsequently, after a control contraction with high K^+ (80 mM), the muscles

were activated with αβ-methylene ATP (a $P2X_1$ purinoceptor agonist (Bo and Burnstock, 1995), 100 nM to 10 μM) for 1 min at each concentration and maximal response was recorded. The tissues were allowed to relax Krebs-Ringer solution for a 5 min wash period between each contraction.

In separate series of experiments in **Paper II**, we used pharmacological blockers to analyze the relative contribution of Rho-kinase and PKC pathways in force development of hypertrophied urinary bladder in comparison with the sham-operated control bladders. To block Rho-kinase and the PKC pathway we used Y27632 (Ishizaki et al., 1996) and GF109203X (Coultrap et al., 1999) that inhibits these two enzymes respectively. After the equilibration period and the initial activation with high K^+, the muscle was activated with carbachol (10 μM, 5 min). Thereafter, the preparations were incubated with Y27632 (10 μM) or GF109203X (1 μM) for 30 min followed by a second challenge with carbachol (10 μM, 5 min) were the peak and sustained force was recorded. The effect of blockers on force was analyzed relative to the initial carbachol contraction (peak and sustained force values) in the absence of drugs.

3.3.3 PKC-induced contractions, effects of blebbistatin (Paper III)

When we performed the experiments for Paper II we observed that a significant PKC induced contractile response was present in the hypertrophic urinary bladder smooth muscle. This finding led to further experimental studies in **Paper III**. The outline of the experiments is illustrated in Figs 3 and 4 of Paper III. We first examined the sensitivity to PDBu (an activator of PKC, Arcoleo and Weinstein, 1985) in control preparations and found that 1 μM PDBu gave a significant inhibition of active force and that 100 nM gave a small potentiation. The latter PDBu concentration was chosen for comparing control and hypertrophic bladders. We used muscarinic stimulation CCh (10 μM, 5 min) were the peak and sustained force values were recorded and activation with 100 nM PDBu (for 15 min). To determine if the PDBu induced contraction was dependent on ROCK, the tissue was incubated for 30 min with Y27632 (10 μM). In separate set of experiments in **Paper III**, we examined the PKC induced contraction in relation to nonmuscle myosin. The preparations were incubated 30 min with blebbistatin (10 μM, an actomyosin inhibitor with higher affinity for nonmuscle myosin compared to smooth myosin, Limouze et al., 2004; Zhang et al., 2017) or DMSO control followed by activation with high K^+ and CCh. The preparations were subsequently activated with PDBu (100 nM) and the force values after 15 min was determined.

3.3.4 Effects of metabolic inhibition with rotenone (Paper IV)

In **Paper IV** we studied the effects on smooth muscle contraction of metabolic inhibition in aorta and urinary bladder preparations. To introduce a partial metabolic inhibition we applied rotenone, a blocker of complex I in the respiratory chain of the mitochondria (Palmer et al., 1968). The following standard protocol was applied for the *in vitro* force recordings: (1) the muscle preparations were first challenged with 2-3 high K^+ control contractions (80 mM, 5 min), (2) followed by 30 min incubation with rotenone (10 μM) or corresponding volume of

DMSO in controls. (3) After the incubation period, the preparations were again activated with high K^+ followed by agonist stimulation; phenylephrine (10 μM, aorta, 5 min) or carbachol (10 μM, urinary bladder, 5 min) and subsequently with PDBu (1 μM, aorta, 20 min). Each contraction was followed by a washout period were the preparations were allowed to relax in Krebs-Ringer solution for 5 min. Rotenone or DMSO for controls were replenished between each wash period. Maximal active force was evaluated relative the initial maximal K^+ (80 mM) peak response.

To further examine different potential mechanisms underlying the reduced force in the rotenone treated tissue (**Paper IV**), we systematically targeted potential cellular mechanisms with blockers/activators and applied the standard protocol as described above. We focused on the following cellular targets: ATP-dependent K^+ channels: glibenclamide (10 μM, Zünkler et al., 1988); small conductance K^+ channels (SK channels): NS8593 (10 μM, Strøbaek et al., 2006); large conductance K^+ channels (BK channels): penitrem (1 μM, Asano et al., 2012); AMP-kinase: inhibitor dorsomorphin (10 μM, Pyla et al., 2014), activator AICAR (1 mM, Davis et al., 2012; Pyla et al., 2014). For each compound effects of respective solvent control were examined in parallel.

To examine if the endothelial- and NO-induced relaxation was affected by rotenone treatment (**Paper IV**), aorta preparations was incubated with (10 μM) rotenone or DMSO control, as described above, and subsequently activated with a submaximal dose of phenylephrine (1 μM) until a stable level of contraction was reached after about 20 min. Acetylcholine was then added (at the stable plateau of the phenylephrine contraction) cumulatively (10 nM, 100 nM, 1 μM) every 2-4 min followed by the addition of SNP (sodium nitroprusside, a nitrous oxide donor activating cGMP-induced relaxation in smooth muscle, Levy, 2005) as a single dose (10 μM) or cumulatively (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM) every 1-2min.

3.3.5 Direct nerve stimulation, (Paper II)

Smooth muscle preparations of the control and hypertrophic urinary bladders were analyzed by direct nerve stimulation (**Paper II**). The preparations were obtained, dissected and mounted in open organ bath as described in the previous sections (**3.1, 3.2** and **3.3.1**). We used electric field stimulation of the samples applying 0.5 ms pulses at different frequencies (1, 5, 10, 15, 20, 25, 30, 40 and 50 Hz) during 5 s at supramaximal voltage. We applied scopolamine (1 μM) to block muscarinic receptors, αβ-methylene ATP (1 μM) to block purinergic ($P2X_1$) receptors and Tetrodotoxin (TTX, 1 μM) to block all nerve-induced contractions (Na^+-action potentials) and evaluated the contraction due to direct activation. We could thus examine the relative role of each neurotransmitter pathway in the nerve induced responses.

3.3.6 Measurement of the Ca^{2+} sensitivity, (Paper II, IV)

The extracellular calcium sensitivity (**Paper II**) of contraction was determined by incubating the muscle for 15 min in Ca^{2+} free N-Krebs solution containing 1 μM of αβ-methylene ATP and scopolamine. Then the bladder strip was subjected to high K^+ (80 mM) depolarizing the

tissue. Every 5 min the extracellular Ca^{2+} concentration increased cumulatively (from 0 to 5 mM) by adding $CaCl_2$ directly into the bath solution. To determine the effect of Rho-kinase on the Ca^{2+} sensitivity the experiments was performed in the presence and absence of Y27632 (10 μM).

For the rotenone treated smooth muscle strips (urinary bladder) in **Paper IV**, we also determined if the calcium sensitivity of the tissue was affected. The protocol was essentially as described above.

3.4 MEASUREMENT OF O_2 CONSUMPTION, PAPER IV

The oxygen consumption (**Paper IV**) in relaxed muscle strips from the urinary bladder was determined using an oxygen electrode essentially as described by Arner et al. (1990). Since we were unable to control the CO_2 in these experiments, the tissue preparations were allowed to equilibrate for 45 min in a MOPS solution gassed with air, composition in mM: NaCl 118, KCl 5, $MgCl_2$ 1.2, Na_2HPO_4 1.2, MOPS 24, glucose 10, $CaCl_2$ 1.6 (pH of 7.4 at 37ºC). Control experiments were made verifying that the rotenone inhibition of force was similar in MOPS and Krebs-Ringer solution. The muscle strips were incubated for 30 min with rotenone (10 μM) or DMSO in control. Next the tissue was mounted with silk threads to a glass holder and stretched to optimal length, held in a 1.3 ml glass chamber that was coupled with an oxygen electrode (Clark electrode, MLT1120, ADInstruments Ltd, Oxford, UK). The glass chamber contained a magnetic stirrer and before each experiment started the Clark electrode in the glass chamber was calibrated using air and N_2. For each preparation, during a 10 min period, the decrease in oxygen content in the glass chamber was recorded. After each experiment the muscle strip was weighed, and the oxygen consumption was related tissue wet weight, thus expressed in μmol min^{-1} $gram^{-1}$.

3.5 CHEMICAL PERMEABILIZATION AND STUDIES OF SKINNED SMOOTH MUSCLE FIBERS, PAPER II

We performed studies on chemically permeabilized (skinned) urinary bladders (**Paper II**). In these preparations the PKC and ROCK pathways and sarcoplasmic reticulum are removed, while the contractile machinery and MLCK/MLCP remain. The purpose was to determine if the increased Ca^{2+} sensitivity of the obstructed urinary bladders was due to altered sensitivity to Ca^{2+} of the contractile machinery.

Preparations of the bladder smooth muscle were permeabilized with 1% Triton X-100 (a detergent) and examined according to Arner and Hellstrand (1985). Initially the preparations were incubated for 30-60 min in a low Ca^{2+} solution with high K^+ (to mimic intracellular environment) containing sucrose to maintain osmolarity (in mM: EGTA 5, KCl 50, sucrose 150 and TES buffer 30, pH 7.4). Then, the tissue was incubated for another 4 h in the same solution with the addition of 1% Triton X-100, to permeabilize the membranes. Finally, the

tissue was rinsed to remove Triton and transferred to a relaxing (low Ca^{2+}, ATP containing solution, with glycerol to prevent freezing and improve permeabilization) solution (in mM: EGTA 4, $MgCl_2$ 10, ATP 7.5, DTE (dithioerythritol) 0.5, TES 30, pH 6.9 and 50 % glycerol and stored at -15°C until further analysis.

The chemically permeabilized bladder preparation were mounted with aluminum clips at each end and attached between two tungsten wire hooks, one connected to an AME force transducer (SensoNor, Horten, Norway) and the other to a micrometer screw (for length adjustment). The tissue was and held horizontally in a small bath (200 µL) at constant stirring and containing solutions with the following composition (in mM): 30 TES buffer, 4 EGTA, 2 Mg^{2+}, 3.2 MgATP, 12 phosphocreatine, 150 KCl and 0.5 µM calmodulin and 0.5 mg/ml creatine kinase, adjusted to pH 6.9. Relaxation and contraction solutions was prepared by altering the free [Ca^{2+}], determined by the ratio of CaEGTA/EGTA in the solution. The relaxation and contraction solutions had 10^{-9} M (pCa 9) and $10^{-4.3}$ M (pCa 4.3) of free Ca^{2+} concentrations respectively and intermediate Ca^{2+} concentration was achieved by mixing these two buffers. The active force at increasing free [Ca^{2+}] was determined and expressed relative to the maximal force at $10^{-4.3}$ M of free [Ca^{2+}].

3.6 REAL TIME QUANTITATIVE PCR, PAPER I

Real time quantitative PCR (qPCR) was performed in **Paper I** to determine if key components in metabolic pathways differed between fast and slow smooth muscle types. The mRNA from different smooth muscle tissues (aorta, femoral artery, ileum and urinary bladder), were extracted with RNeasy Kit (Qiagen). The purity and concentrations of the mRNA was determined by measuring in a NanoDrop spectrophotometer at 260/280 nm and at 260/230 nm (acceptable values >1.9 and >2.0 respectively). The cDNA of the metabolic enzymes was generated with High Capacity Reverse Transcription Kit (Applied Biosystems) and analyzed in duplicates on 96-well plates with RT-qPCR (quantitative real time PCR) technique using Fast SYBR Green MasterMix and a real-time PCR from Applied Biosystems. All primers used are presented in Table 1 of Paper I. Hypoxanthine-guanine-phosphoribosyl transferase (HPRT) was used as housekeeping gene. We used the relative standard curve method with pooled samples from all tissues as standard and normalized to HPRT (similar to delta-delta Ct method). All mRNA values in diagrams of Paper I are expressed relative to the standard normalized to HPRT.

We also determined the mRNA expression of the inserted myosin heavy chain (SM-B) using regular PCR. In this case the primers result in products with different size depending on the presence (SM-B) or absence (SM-A) of the base pairs corresponding to the extra 7 amino acid insert in SM-B. The products were separated on agarose gels and the relative expression of SM-B (evaluated as larger product band/sum of both bands) was used as a marker for expression of fast smooth muscle myosin.

3.7 QUANTITATIVE WESTERN BLOT ANALYSIS, PAPER I, II & III

In **Paper I, II** and **III** quantitative Western blot analysis was used to determine different cell signaling and contractile proteins in different smooth muscle tissues; **Paper I**: aorta, mesenteric artery, ileum and urinary bladder; **Paper II** and **III**: normal and hypertrophic urinary bladder.

Isolated smooth muscle tissues were placed in a precooled (liquid nitrogen) mortar. The tissue was thoroughly pulverized and then dissolved in homogenizing buffer; 1 % SDS, 1 mM Na_3VO_4 and 1 % PMSF (phenylmethylsulfonyl fluoride). Determination of the protein concentration was made using a protein assay from Bio-Rad (Richmond, CA). Then, samples were separated using SDS-PAGE, loading equal amount of protein from each sample, using a MiniGel system (Bio-Rad). Proteins from each gel were blotted onto nitrocellulose membranes and stained with a primary and a secondary antibody, subsequently visualized with enhanced chemiluminescence kit (ECL, Amersham Bioscience) and analyzed using Quantity One software from Bio-Rad. Eight different primary antibodies were used in the studies (**Papers I, II**): rabbit PKC (Santa Cruz Biotechnology Inc., California, USA), rabbit CPI17 (Upstate, Lake Placid, New York, USA), rabbit PP1β (Calbiochem, Darmstadt, Germany), mouse RhoA (Santa Cruz), rabbit RhoGDI (Santa Cruz), goat ROCK1 (Santa Cruz), goat ROCK2 (Santa Cruz), goat MYPT-1 (Santa Cruz). For **Paper III**: rabbit SMemb (nonmuscle myosin B, from Drs I. Morano and H. Haase, Berlin). In Western blot analysis the intensity of the ECL signal for each sample was normalized to the signal from the normal urinary bladder samples.

3.8 IMMUNOHISTOCHEMISTRY, PAPER III

Immunohistochemistry was performed in **Paper III** to visualize the localization of nonmuscle myosin and estimate if the expression is altered in obstructed relative to sham-operated urinary bladders. Urinary bladders were fixed in 4% paraformaldehyde (PFA) and frozen sections were made. A pre-conjugated smooth muscle alpha actin antibody was used to visualize the smooth muscle layer in the tissue sections. Nonmuscle myosin was visualized by staining with a primary antibody SMemb and a fluorescent secondary antibody. Images were obtained with a confocal microscope (Zeiss LSM510) and analyzed with LSM Image Browser (Carl Zeiss AG, Oberkochen, Germany) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Table 1. Pharmacological substances (blockers and activators) used in the different papers.

Compound	Cellular target/action	Tissue (from mouse)	Paper
α,β-mATP	α,β-methylene ATP. Agonist and desensitizer of P_2X_1 purinoceptors. A more stable analog of ATP.	Urinary bladder	II
Acetylcholine (ACh)	Acetylcholine binds to muscarinic receptor and induce endothelium-dependent relaxation of the smooth muscle.	Aorta	IV
AICAR	Activator of "AMP-activated protein kinase" (AMPK).	Aorta Urinary bladder	IV
Blebbistatin	Inhibitor of actomyosin with a weak affinity smooth and stronger for nonmuscle myosin II.	Urinary bladder	III
Carbachol (CCh)	Cholinergic agonist. Activates the acetylcholine receptors.	Urinary bladder	II, III, IV
DMSO	Dimethyl sulfoxide. Anhydrous solvent used to dissolve pharmacological substances (blockers and activators).	Aorta Urinary bladder	II, III, IV
Dorsomorphin	"Dorsomorphin dihydrochloride" also known as "Compound C". Potent inhibitor of AMPK.	Aorta Urinary bladder	IV
GF109203X	A potent and selective competitive inhibitor of Protein Kinase C (PKC).	Aorta Urinary bladder	II, III
Glibenclamide	Blocker of ATP-sensitive K^+ channels.	Aorta Urinary bladder	IV
NS8593	Blocker of SK channels "small conductance Ca^{2+}-activated K^+ channels".	Aorta Urinary bladder	IV
PDBu	Phorbol 12,13-dibutyrate. Activates protein kinase C (PKC).	Aorta Urinary bladder	II, III, IV
Penitrem A	Blocker of BK channels "large conductance Ca^{2+}-activated K^+ channels".	Aorta Urinary bladder	IV
Phenylephrine (Phe)	Agonist of α_1-adrenoceptors.	Aorta Urinary bladder	IV
Rotenone	Inhibitor of complex I in the electron transport chain of the mitochondria.	Aorta Urinary bladder	IV
Scopolamine	Scopolamine hydrobromide. Non-selective muscarinic antagonist.	Urinary bladder	II
SNP	Sodium nitroprusside. NO-donor that activates guanylyl cyclase (GC), and thus can induce relaxation of the smooth muscle independent of the endothelium.	Aorta	IV
TTX	Tetrodotoxin. Blocks nerve transmission. Binds to the voltage-gated sodium channels thus inhibits action potentials in neurons.	Urinary bladder	II
Y27632	Inhibitor of ROCK "Rho-associated protein kinase".	Urinary bladder	II, III

4 RESULTS AND DISCUSSION

4.1 SMOOTH MUSCLE CONTRACTILE KINETICS CORRELATES WITH SM-B EXPRESSION

Smooth muscle is a heterogenic tissue with a large span in contractile, metabolic and regulatory properties (section **1.5**). No clear characterization of "smooth muscle types" is however available today. Therefore, in the first study (**Paper I**), we focused on the complexity of smooth muscle characteristics aiming at developing a more comprehensive view of smooth muscle types.

Since smooth muscle, like all muscles, converts metabolic energy to contractile function in a regulated process and exhibits a large span (about 5-7-fold difference) in contractile kinetics, it seems logical to divide the smooth muscle according to the contractile kinetics, similar to the division of skeletal muscle, i.e. fast (type II) and slow twitch (type I). In the skeletal muscle, several contractile and structural parameters and expression of regulatory, metabolic, cytoskeletal and contractile proteins differ between the muscle types (Schiaffino and Reggiani, 2011). In smooth muscle tissues also vary in the intracellular signaling (i.e. activation/deactivation). Also, although the smooth muscle is generally considered an economical tissue, a more detailed metabolic picture of this diverse tissue is missing. It could thus be argued that the a more complete characterization the smooth muscle could involve consideration of **contractile**, **cell signaling** and **metabolic parameters**.

In **Paper I** we chose a set of smooth muscles from the mouse, exhibiting a large difference in contractile kinetics: the urinary bladder, intestinal muscle, muscular arteries and aorta. Our starting point for the division was the maximal shortening velocity (V_{max}). This parameter is considered to reflect the kinetics of the acto-myosin cross-bridge turnover, and to be rate-limited by the rate of ADP release after completion of the power stroke (cf. Arner and Malmqvist, 1998; Löfgren et al., 2001;). The V_{max} values have been determined in our laboratory for several of these smooth muscles and data can also be found in the literature. Variation in the values can be dependent of species, experimental temperature, mode of activation (skinned or intact muscle), and the method used. Approximate V_{max} values at 20-22 ^{o}C in muscle lengths per second (ML/s) were for aorta: Löfgren et al., 2001 (0.015 ML/s, guinea pig), Rhee et al., 2006 (0.07 ML/s, mouse); muscular artery: Davis and Davison, 2002 (0.02 ML/s, mouse caudal artery, corrected for temperature); intestine: Risse et al., 2012, (0.09 ML/s, mouse ileum , corrected for temperature), Löfgren et al., 2002 (0.11 ML/s, guinea pig), Malmqvist and Arner, 1991 (0.20 ML/s, taenia coli guinea pig); urinary bladder: Scott et al., 2008 (0.19 ML/s mouse). When reviewing the available data, a clear picture emerges, where the aorta belongs to a "slow" group whereas the urinary bladder would be "fast". It is likely that intermediate groups exist (cf. Malmqvist and Arner, 1991), but in our study we chose to initially screen 4 smooth muscle tissues and measure several signaling and metabolic parameters. The analysis was mainly focused on the fast bladder and the slow

aorta, and we expect that our identification of the main characteristics of these two extremes can be used in the future to screen a larger number of smooth muscles possibly different species with fewer parameters, to directly answer the question if intermediate groups exist.

Table 2. Smooth muscle tissues exhibit a large span in contractile kinetics (shortening velocity, V_{max}). Relative SM-B expression correlates with shortening velocity and is thus a marker for the contractile system.

Tissue	Organ	Type	V_{max}	SM-B
Aorta	Large elastic artery	Slow	0.015-0.07	0.07
Femoral/ Mesenteric artery	Muscular artery	Slow	0.02	0.11
Ileum	Visceral	Fast / Intermediate	0.09-0.20	0.59
Urinary bladder	Visceral	Fast	0.19	0.91

The myosin isoform expression is one factor that determines the contractile kinetics of the smooth muscle, and the inserted myosin heavy chain (SM-B) has been reported to translocate actin at a higher velocity (Kelley et al., 1993) compared to the non-inserted variant (SM-A). It is clear that also the essential light chain isoform (LC_{17}) composition correlates with V_{max} (Malmqvist and Arner, 1991) and that nonmuscle myosin is expressed and can influence the kinetic in the slow aorta (Rhee et al., 2006). We used the relative expression of SM-B (determined with PCR) as a marker for contractile kinetics and we first determined the expression of the inserted myosin heavy chain (SM-B) in the selected tissues using PCR. As illustrated in **Table 2** a strong correlation exists between SM-B expression and V_{max} values (extracted from the literature above, cf. Arner et al., 2003) for the different smooth muscle tissues. Thus, in **Paper I** SM-B is used as a marker for kinetics of the contractile system and high expression relates to a fast contractile type. The urinary bladder represents a fast contractile type, the aorta and arteries slow smooth muscles and the intestine possibly an intermediary type.

4.2 DIFFERENCE IN METABOLIC PATHWAYS BETWEEN FAST AND SLOW SMOOTH MUSCLE

We displayed the metabolic and signaling parameters in relation to the SM-B expression (cf. **Table 2**, used in all Figures in **Paper I**, and in **Figs 8** and **9** below). We applied qPCR (real time quantitative PCR) using primers from 15 different metabolic enzymes and factors (primers mainly from Sjögren et al., 2007, listed in Table 1 in Paper I) to address the aim if

the metabolic components were related to the contractile kinetics. We chose metabolic components for different pathways in the energy metabolism (section **1.6**). We found that components of glucose uptake pathway were more expressed in the slow aorta smooth muscle. The insulin independent GLUT1 and hexokinase were markedly higher expressed in the slower compared to the faster tissues (**panel A** of **Fig. 8** below and Fig. 3 in Paper I). Our measurements relate to the mRNA expression and if these values are correlated with high protein expression and activity the data would suggest that the slow muscles have a larger capacity for glucose uptake. Higher expression of GLUT1 has been correlated with higher glucose uptake (Kaiser et al., 1993) in smooth muscle and the hexokinase is the initial and rate limiting step in glucose uptake (Lehninger et al, 2000). Although the slow vascular smooth muscle would have a more economical mode of contraction with low ATP turnover (Arner and Hellstrand, 1981), these tissues are required to be contracted for longer periods during sustained tension maintenance in the vascular wall. It is likely that a continuous support of energy is physiologically important to enable long contractions, when cellular energy stores might be depleted. A consequence of an active insulin independent glucose uptake and efficient glucose metabolism might also be that the arterial muscles are more sensitive to high extracellular glucose levels, providing a potential contributing factor for vascular disease in diabetes/metabolic syndrome (Petrie et al., 2018). Interestingly the slow vascular tissues also had high expression of PEPCK (Phosphoenolpyruvate carboxykinase), which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, an initial step in gluconeogenesis (**panel B** of **Fig. 8** below). Although the function of this pathway is unclear at present the expression of PEPCK appears to be a prominent marker for a slow smooth muscle. The expression of LDH (lactate dehydrogenase) was similar in the different tissues. However, it has previously been observed that the slow aorta expresses more of the H-form of LDH (less prone to produce lactate) compared to the faster urinary bladder smooth muscle (Malmqvist et al., 1991a). The faster bladder muscle can decrease the expression of the H-form and changes towards a slower phenotype during hypertrophy (Malmqvist et al., 1991a; Sjuve et al., 1996), showing that LDH isoform expression is not simply correlated with the contractile kinetics, but might be associated with other metabolic demands. G6PDH (Glucose-6-phosphate dehydrogenase) is a key enzyme in the pentose phosphate pathway generating NADPH which is important for protection against oxidative stress. Deficiency in G6PDH expression is correlated with cardiovascular disease and decreased cellular defense against oxidative stress (Hecker et al., 2012). A negative correlation between G6PDH activity and expression of contractile proteins has been reported (Chettimada et al., 2016). Although speculative, the lower expression in the urinary bladder might reflect a higher sensitivity to oxidative stress and promote the maintenance of a contractile phenotype.

AMP-activated protein kinase (AMPK) is a metabolic sensor activated in conditions of high AMP/ATP ratios (Hardie, 2011). The enzyme has several targets and in smooth muscle it has been reported to induce a relaxation of vascular smooth muscle via inhibition of PKC (Davis et al., 2012). In skeletal muscle it promotes glucose uptake and AMPK is a main target for the antidiabetic compound metformin (Zhou et al., 2001). We report a slightly higher expression

of AMPKα2 isoform in the faster smooth muscles, which does not correlate with the relaxant effect of AICAR, which is most prominent in the aorta. However as reported in **Paper IV** of this study, AMPK might have a protective action under ischemic conditions and a higher expression can be important in this action in the urinary bladder (cf. Section **4.8**).

Major differences between the fast and slow smooth muscles were observed in the expression of enzymes involved in lipid synthesis (FAS: Fatty acid synthase, ACC2: Acetyl-coenzyme A carboxylase beta) and hydrolysis (HSL: hormone sensitive lipase, LPL: lipoprotein lipase). The expression patterns are shown in Fig. 5 of Paper I and exemplified in **panel C** of **Fig. 8**, below. These enzymes were significantly higher expressed in the slow smooth muscle, which suggests an enhanced lipid turnover. The higher FAS and ACC2 in slow muscle would promote fatty acid synthesis. Interestingly, ablated ACC2 in mice leads to a resistance to obesity-inducing diets with less weight gain and reduced fatty acid accumulation (Wakil and Abu-Elheiga, 2009). A detailed analysis of these lipid metabolism pathways was not the aim of **Paper I** and it is presently unclear how the differences in these enzymes are related to cellular metabolism in fast and slow smooth muscle. However, but these lipid metabolism enzymes are clearly strong markers for the smooth muscle phenotype.

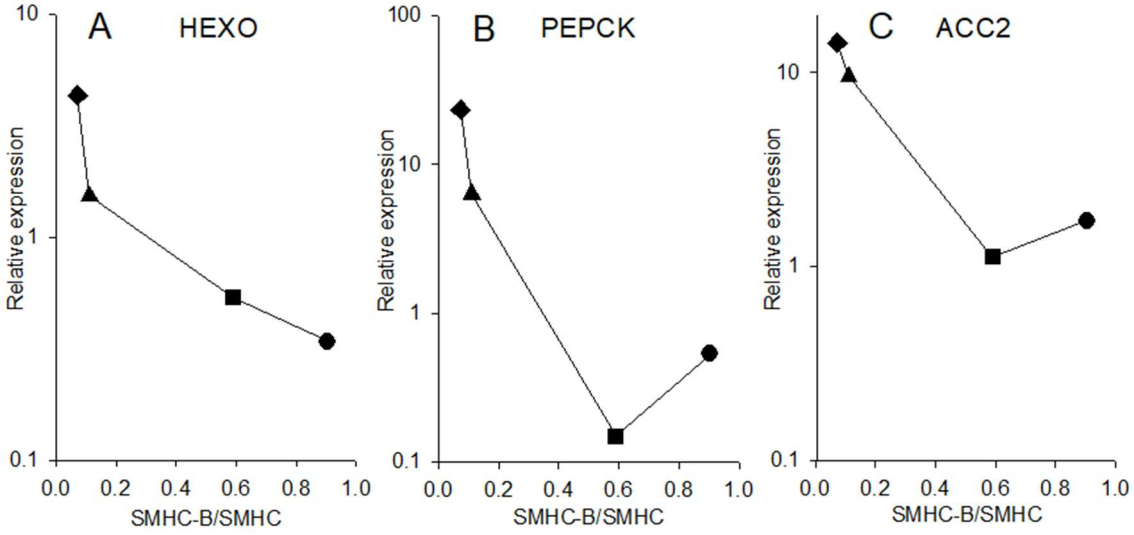

Figure 8. Expression of key metabolic components in smooth muscles with different kinetics. Aorta (diamond), muscular artery (triangle), ileum (square) to urinary bladder (circle). The expression of mRNA for the respective enzyme is related to the contractile kinetics evaluated by the relative expression of inserted myosin heavy chain. (SM-B). Figure modified from Figs 3-5 in **Paper I**.

4.3 DIFFERENCE IN CELL SIGNALING PATHWAYS BETWEEN FAST AND SLOW SMOOTH MUSCLE

In a second part of **Paper II** we used quantitative Western blot analysis to determine different cell signaling proteins from the main Ca^{2+} sensitizing pathways (RhoA, PKC, MLCP)

focusing on 8 different proteins. The main finding was that the slower muscles (aorta, muscular artery) had low expression of the phosphatase subunit MYPT1 (**panel A** of **Fig. 9**), higher expression of the ROCK1 isoform (**panel C** of **Fig. 9**) and higher expression of CPI17 (**panel B** of **Fig. 9**, mainly in the aorta). The catalytic subunit (PPIβ) was not different between tissues, but the expression of the targeting subunit MYPT1 was clearly different with a lower expression in the slower smooth muscles (**panel A** of **Fig. 9**, below). It has been shown that low expression of MYPT1 is correlated with a lower phosphatase activity and a generally higher Ca^{2+} sensitivity (Ekman et al., 2005). A low phosphatase activity has previously been reported for tonic compared to phasic smooth muscles (Gong et al., 1992). This seems to be coordinated with higher expression of components of both RhoA pathway (ROCK1) and the main target for PKC (CPI17). Expression of the latter protein is most likely related to increased PKC induced sensitization (Woodsome et al., 2001), and to the sustained and significant PDBu-induced contraction observed in the aorta (e.g. Fig. 1 panel A of **Paper IV**). The slow muscle thus also appears to have a higher capacity for Ca^{2+} sensitization via receptor activation and a slower relaxation.

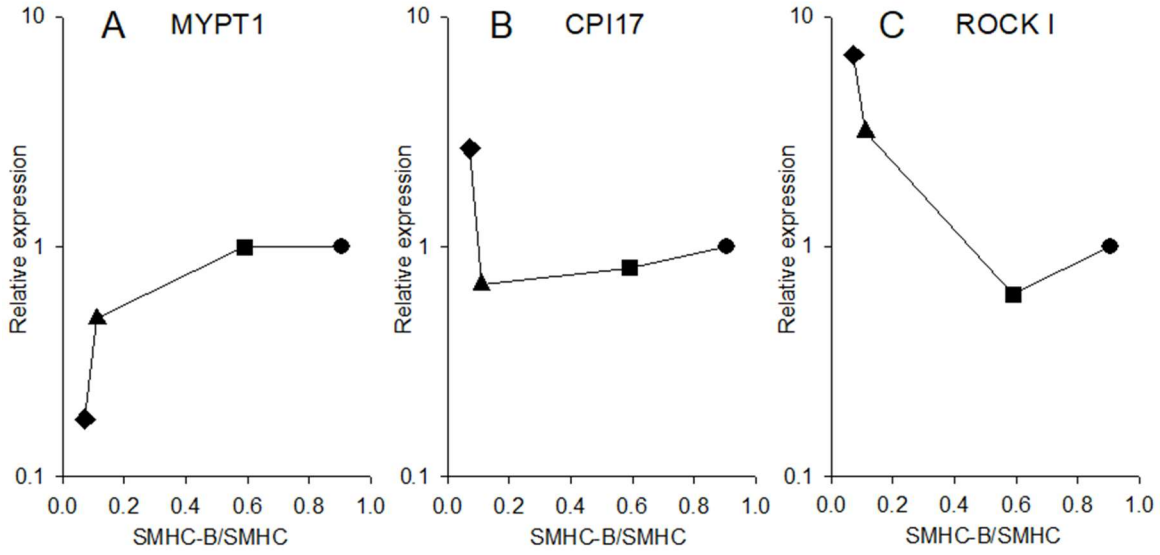

Figure 9. Expression of key cell signaling components in smooth muscles with different kinetics. Aorta (diamond), muscular artery (triangle), ileum (square) to urinary bladder (circle). The protein expression for the respective cell signaling component is related to the contractile kinetics evaluated by the relative expression of inserted myosin heavy chain. (SM-B). Figure modified from Figs 1-2 in **Paper I**.

It should be stressed that our analysis in **Paper I** was focusing on identifying key markers for fast and slow smooth muscles, based on Western blot analysis and qPCR. Although protein and mRNA expression most likely are associated with protein/enzyme activity, we cannot at present definitively show that our measurements reflect functional differences. A logical next step for future studies would be to choose 2-3 metabolic enzymes that display a major

difference in mRNA and protein expression between fast and slow smooth muscle and measure the expression and activity of these enzymes in a broader range of smooth muscle tissues.

Table 3. Main characteristics of fast and slow smooth muscle types

	Properties of the smooth muscle	"fast" smooth muscle e.g. urinary bladder		"slow" smooth muscle e.g. aorta	
Contractile	Shortening kinetics	↑	high V_{max}*	↓	~5 times slower*
	Tension development	↑	high rate of tension development*	↓	~2 times slower*
	Myosin types	↑	high LC17a*	↓	~16 times lower*
		↑	high SMHC-B	↓	~12 times lower
		↓	low nonmuscle myosin*	↑	higher nonmuscle myosin*
	ADP dependence	↓	low MgADP affinity*	↑	~36 times higher affinity*
	ATP dependence	↑	high MgATP affinity*	↓	~4 times lower affinity*
	Phosphate dependence	↑	high P_i inhibition of force*	↓	~2-4 times lower P_i inhibition*
Metabolic	Glucose uptake and glycolysis	↓	lower insulin independent GLUT1	↑	~3 times higher
		↔	no difference in insulin dependent GLUT4	↔	no difference
		↓	low hexokinase	↑	~13 times higher
		↔	PYRK no difference	↔	no difference
	Lactate production	↔	LDH no difference	↔	no difference
		↓	low LDH-H*	↑	~1.4 times higher*
	NADPH synthesis	↔	G6DPH no difference	↔	no difference
	Gluconeogenesis	↓	low PEPCK	↑	~43 times higher
	Mitochondrial signalling	↔	TFAM no difference	↔	no difference
	Metabolic sensing	↔	AMPK1 no difference	↔	no difference
		↑	high AMPK2	↓	~5 times lower
	Lipid metabolism	↓	low HSL	↑	~10 times higher
		↓	low LPL	↑	~13 times higher
	Lipid synthesis	↓	low FAS	↑	~6 times higher
		↓	low ACC2	↑	~8 times higher
		↔	MCD no difference	↔	no difference
Cell signaling	Dephosphorylation of myosin	↑	high expression of targeting subunit, MYPT1	↓	~6 times lower
		↔	no difference catalytic subunit PP1β	↔	no difference
	Protein kinase C pathway	↓	low CPI17	↑	~3 times higher
		↔	PKCα no difference	↔	no difference
	Rho-Rhokinase pathway	↓	low ROCK1	↑	~7 times higher
		↔	no difference in ROCK2, RhoA and RhoGDI	↔	no difference

* Data obtained from the literature. Table modified from Table 3 in **Paper I**.

4.4 MAIN PROPERTIES OF FAST AND SLOW SMOOTH MUSCLE

In a summarizing **Table 3** modified from Table 3 in **Paper I** we attempted to make a more systematic characterization of slow and fast smooth muscle types according to the main parameters (**contractile**, **metabolic**, and **cell signaling**). In general, a slow smooth muscle, e.g. found in large arteries, have slow cross-bridge turnover with low shortening velocity and high tension economy which is correlated with expression of proteins associated with slow acto-myosin turnover (low SM-B, high LC_{17b} and high expression of nonmuscle myosin). It has slow kinetics in its deactivation (low phosphatase) and high expression of components on Ca^{2+} sensitizing pathways and would thus be able to modulate its contraction via receptor activation. Its energy consumption is generally lower than in faster smooth muscles, but its ability to take up glucose as well as lipid turnover is high, possibly related to the requirement of sustained energy supply and possibly also related to an increased sensitivity to extracellular glucose levels.

4.5 PARTIAL URINARY OUTLET OBSTRUCTION INDUCES HYPERTROPHIC GROWTH

Paper I, discussed above, identified key properties of fast and slow smooth muscle. It can also be asked if an individual smooth muscle can change its properties. In different physiological situations and in pathophysiology, smooth muscle can undergo significant adaptation in structure and function. With regard to contractile function, changes in the cross-bridge kinetics towards faster and slower phenotypes have been described in different animal models, e.g. during hypertrophic growth in the urinary bladder (Sjuve et al., 1996; Scott et al., 2008), in asthma in trachea, after thyroxin treatment in intestine (Löfgren et al., 2002). Adaptation and changes in cell signaling is of significant interest since information on these cellular mechanisms can provide a basis for pharmacological therapy of smooth muscle pathologies. As mentioned in the Introduction (**Section 1.8**) alterations in urinary bladder function with the over active bladder syndrome and incontinence are common and introduce significant burden to the affected persons and to our society. Research and therapeutic initiatives are extensive but yet not fully successful (Andersson, 2016). In **Paper II** we addressed adaptation in cell signaling properties including both PKC and RhoA signaling pathways in a mouse model for urinary bladder hypertrophy/overactivity, resembling changes in urinary outflow occurring e.g. after prostatic enlargement. We used a mouse model (Pandita et al., 2000; Sjuve et al., 1995; Scott et al., 2008) to induce hypertrophic growth of the urinary bladder by ligating the urethra to induce a partial urinary outlet obstruction that gave a significant increase of urinary bladder weight. The obstructed and sham-operated animals had no difference in animal weight gain. There was no change in general animal behavior or animal wellbeing. A normal adult mouse bladder (sham operated), weighed about 20 mg and the partially outflow obstructed bladders increased in weight more than 3 times to about 65 mg in about 2 weeks. The increase in urinary bladder weight was associated with changes in contractile protein (actin and myosin) and with a relative increase in cytoskeletal

proteins (intermediate proteins desmin/vimentin) similar to that described previous for mouse and rat (Malmqvist et al., 1991b; Scott et al., 2008).

4.6 INCREASED SENSITIVITY TO CHOLINERGIC STIMULATION IN HYPERTROPHIC BLADDER

A first step in **Paper II** was to examine the physiological regulation of the control and the hypertrophic bladder detrusor via direct activation if the intramural nerves. The samples were stimulated using electrical field stimulation at different frequencies. Increased frequency gave increased active force (see **Fig. 10** below, and Fig. 1 in **Paper II**). By adding scopolamine (a muscarinic receptor blocker) we could identify the relative contribution of the cholinergic nerve component. In controls, scopolamine reduced the nerve induced responses by about 45 % whereas the hypertrophic bladders were more inhibited (by about 70%), suggesting a larger relative contribution from the cholinergic pathway in the hypertrophic bladders.

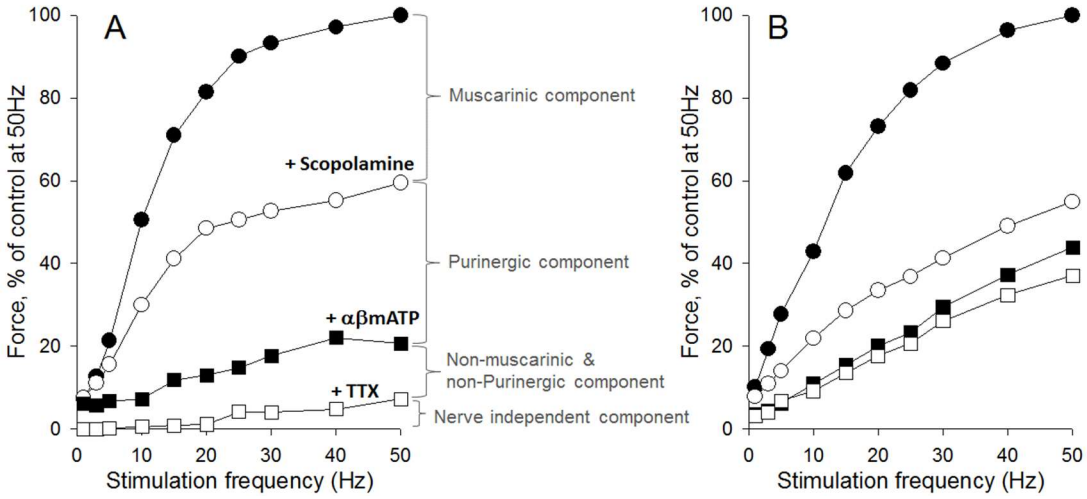

Figure 10. Electric field stimulation. Force response of control (**A**) and hypertrophic (**B**) urinary bladder preparations stimulated at different frequencies after application of blockers of nerve transmission to identify the muscarinic and the purinergic components in the responses; control (filled circles), 1μM scopolamine (white circles), 1μM αβ-mATP (filled squares), 1μM TTX (white squares). Figure modified from Fig. 1 in **Paper II**.

Further addition of α,β-methylene ATP (which desensitizes the $P2X_1$ purinoceptors) reduced the nerve mediated responses of controls significantly, but had only minor effects on the obstructed bladders, suggesting a downregulation of the ATP-dependent contractile activation. Addition of TTX (a nerve blocker) reduced the contractile responses further by about the same amount in both tissues, reflecting a similar and marginal contribution from

non-cholinergic and non-purinergic pathways. The remaining part of the responses to electrical field stimulation was increased in the hypertrophic muscles, reflecting an increased sensitivity to direct electrical stimulation if the bladder muscle. These results thus suggest that the hypertrophic urinary bladder downregulate the purinergic nerve component, upregulate the cholinergic nerve component and increase the sensitivity to direct electrical stimulation.

Since the nerve induced responses suggested changes in reactivity of the hypertrophic muscle, we examined the pharmacomechanical and electromechanical coupling, i.e. after activation with direct depolarization (KCl) or agonists (the muscarinic agonist carbachol and the $P2X_1$ receptor agonist α,β-methylene ATP) in the normal and hypertrophied urinary bladders. Contraction induced by high K^+ is due to the depolarization and opening of voltage-gated Ca^{2+} channels, increasing the intracellular [Ca^{2+}] and triggering contraction by activating the MLCK. Carbachol is a cholinergic agonist, binding to a G-protein coupled receptor in the membrane that triggers a signaling cascade/network of enzymes activating the contractile filaments. The α,β-methylene ATP is considered to activate depolarization of the membrane via the $P2X_1$ receptor opening a cation channel (Surprenant et al., 1995). The *in vitro* application of α,β-methylene ATP did not reveal a difference in the sensitivity between control and hypertrophic bladders (Panel A of Fig. 2 in **Paper II**), suggesting that the alteration in the purinergic nerve component resides in the nerve or nerve terminal rather than on the post synaptic receptor or cellular signaling.

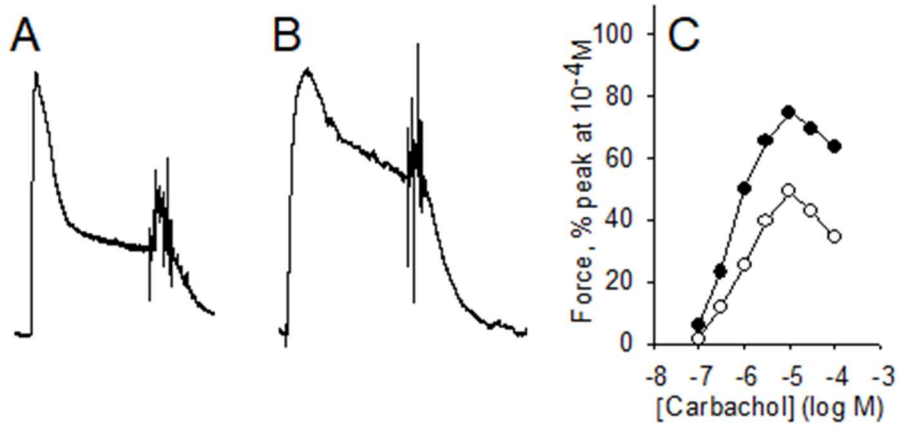

Figure 11. Contractile response to carbachol stimulation. Original recordings in: control (**Panel A**) and hypertrophied (**Panel B**) bladder strips activated with 10 µM carbachol. Note the pronounced sustained contractile component in the hypertrophied smooth muscle. **Panel C**: Force responses of control (open circles) and hypertrophied (filled circles) urinary bladder preparations, activated with the muscarinic agonist carbachol. Figure modified from Fig. 2 in **Paper II**.

We observed an increased sensitivity to carbachol response in the phasic (peak) part of contraction (Panel B of Fig. 2 in **Paper II**) for the hypertrophied smooth muscle, whereas the

tonic (plateau) part of carbachol contractile response gave about 1.5 times stronger (higher) maximal response at unaltered sensitivity to the agonist (**Panel C** of **Fig. 11**; Panel C of Fig. 2 in **Paper II**). These findings are consistent with the result from the direct nerve stimulation.

We examined if the enhanced cholinergic responses of the hypertrophic muscle could be due to an increase in the Ca^{2+} sensitizing pathways. Inhibition of the Rho kinase pathway (with Y27632) inhibited the plateau more than the peak in the control bladders, suggesting that the Rho kinase pathway is more active during the sustained phase of contraction in the urinary bladder. The effect of Y27632 on the hypertrophic bladders was similar, except for a slightly lower sensitivity of the plateau response. Inhibition of PKC (with GF109203x) inhibited force less than inhibition of the Rho pathway and no major differences could be observed between control and hypertrophic bladders (Fig. 3 of **Paper II**). We confirmed that Y27632 and GF109203x also inhibited the more transient nerve induced contractions, to a similar extent in controls and hypertrophic preparations. These results thus suggest that the Rho pathway is recruited to a larger extent than PKC in bladder cholinergic activation. Changes in RhoA ad PKC are however not primarily responsively for the altered responses to in cholinergic stimulation (i.e. in pharmacomechanical coupling) in the hypertrophic urinary bladders. Interestingly, we found an increased sensitivity to $[Ca^{2+}]$ in high K^+ depolarized (i.e. electromechanical coupling) tissues for the hypertrophied urinary bladders that was abolished with Y27632. This suggests that direct influx of Ca^{2+} seems to activate the Rho pathway as suggested by Ratz et al. (2005) or that a constituently active Rho signaling is present and becomes important during Ca^{2+} influx and becomes masked by cholinergic stimulation. Western blot analyses shoved that hypertrophic growth upregulates the RhoA and PKC signaling pathways whereas the MYPT-1 and PP1β were lowered (Fig. 5 in **Paper II** and **Fig. 12,** below). Interestingly, although the urinary bladder is a fast smooth muscle (as discussed in **Paper I**) these characteristics correlates better with a slow smooth muscle type, thus indicating that the hypertrophying urinary bladder undergo a transition from a fast to a slow smooth muscle type, consistent with mechanical data (Scott et al., 2008). We could however not correlate a decreased phosphatase activity to an increased Ca^{2+} sensitivity of the skinned muscle preparations (Panel C of Fig. 4 in **Paper II**), which might suggest that some additional factor is altered during skinning or that a change in phosphatase activity is linked to an increased Rho-kinase pathway as suggested by the Ca^{2+} sensitivity measurements in depolarized tissue.

In conclusion, hypertrophic growth of the urinary bladder increases the sensitivity to muscarinic receptor stimulation, upregulation of components in Rho-kinase and PKC. However, these pathways are not activated to a larger extent after receptor activation in the hypertrophic muscle. Nevertheless, the Rho Kinase pathway is upregulated and contributes to Ca^{2+} sensitization after depolarization in hypertrophic bladders. Based on these results, one can speculate that Rho-kinase inhibition (i.e. Fausodil®) can be a potential therapeutic avenue in treating OAB, reducing the pathological phasic activity (electromechanical coupling) and leaving the receptor mediated (pharmacomechanical coupling) part of contraction less affected.

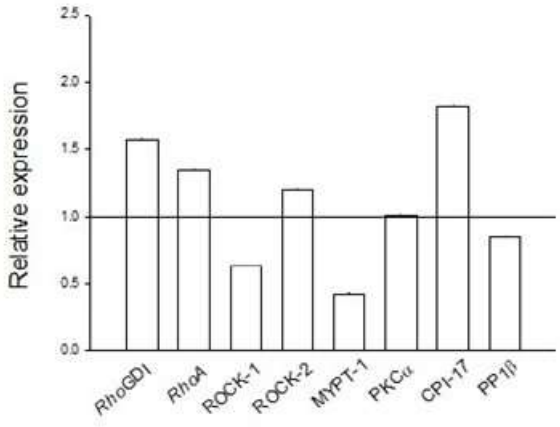

Figure 12. Expression of cell signaling components. Protein content (Western blot) in hypertrophied bladder tissue, relative to the expression in sham operated control bladders (line at 1). Figure modified from Fig. 5 in **Paper II**.

4.7 PKC INDUCED CONTRACTION IS ABOLISHED BY BLEBBISTATIN

When performing the experiments for **Paper II**, we made an interesting observation which initiated further studies in **Paper III**. We found that a prominent contraction could be elicited by direct activation of protein kinase C (PKC) with PDBu (Phorbol 12,13-dibutyrate) in hypertrophied urinary bladder (illustrated in **Fig. 13**, and Fig. 3 and 4 in Paper II). As seen in **Fig. 13** carbachol induced a transient contraction in a control bladder (upper trace) and a more sustained contraction in the hypertrophic bladder (lower trace), as previously also shown in Paper II. The control bladder did not respond to direct PKC activation by PDBu whereas the hypertrophied bladders developed a prominent sustained contraction (lower trace in **Fig. 13**).

Smooth muscle tissue can express nonmuscle myosin in addition to smooth muscle myosin (Morano et al., 2000; Löfgren et al., 2003; Rhee et al., 2006). Nonmuscle myosin heavy chain is expressed from separate genes (nonmuscle myosin A and B, MYH9, MYH10) relative to smooth muscle myosin and can form filaments and generate contraction with slow kinetics (Walklate, 2016). In the urinary bladder, nonmuscle myosin is expressed during fetal life and downregulated early after birth, thus not expressed normally in adult bladder (Löfgren et al., 2003). We examined the role of nonmuscle myosin and protein kinase C in regulation of the PDBu (PKC) induced contraction in the hypertrophic bladders, addressing the possibility that the hypertrophic growth caused development of a separated contractile component with unique activation. Nonmuscle myosin is an interesting candidate since it has been found to be upregulated in the serosa of hypertrophic urinary bladders from the rabbit (Sartore et al., 1999) and in cells of the interstitium of hypertrophied rat bladders (Sjuve et al., 2001). This contractile component thus seems to be structurally separate from the smooth muscle.

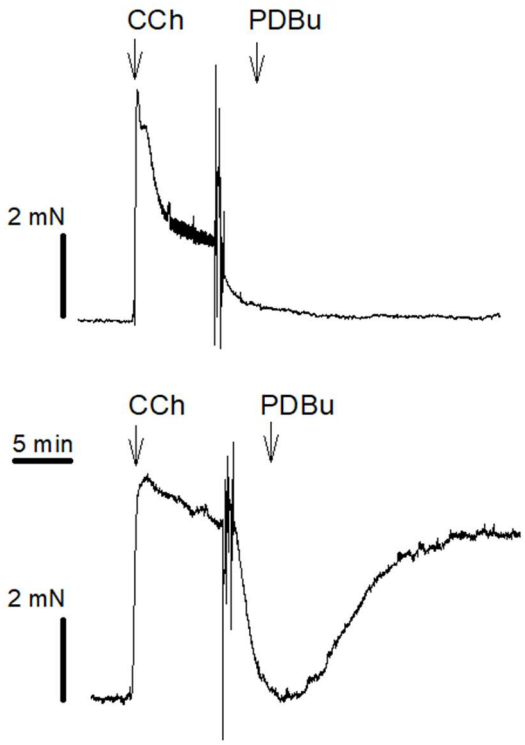

Figure 13. Original force recordings. From control (upper trace) and hypertrophied (lower trace) mouse urinary bladders, first activated with carbachol (CCh) and thereafter with PDBu. Note the substantial force development after PDBu (PKC) activation in hypertrophied urinary bladder, which is not observed in control bladders. Figure modified from Fig. 3 in **Paper III**.

Immunohistochemistry (Fig. 1 of **Paper III**), showed a thicker smooth muscle (smooth muscle alpha actin positive) layer in the wall of the hypertrophied detrusor muscle. The nonmuscle myosin component (stained with SMemb, i.e. nonmuscle myosin B) could be sparsely observed in the wall of the control bladder, but increased markedly in the hypertrophic wall, both in the muscle layer and as a separate component in the serosa. It has been observed that PDBu (PKC activation) can induce a contraction in newborn urinary bladders expressing nonmuscle myosin, suggesting that PKC can activate this contractile component (Lamounier-Zepter et al., 2003). We found in **Paper II** that the PKC activated contractile component is not recruited during normal muscarinic membrane receptor activation, since these contractions in the hypertrophic muscle were not more sensitive to GF109203x (the PKC inhibitor) compared to the responses of the control. This is in contrast to the pronounced contraction elicited by direct PKC activation vid PDBu in the hypertrophic muscle (**Fig. 13** above and Fig. 3 and 4 in **Paper III**). We show that the PDBu induced responses are not affected by Y27632, and therefore do not include activation of the Rhokinase pathway. Currently we do not know which cellular signaling pathway or receptors that can activate this separate PKC dependent contractile component in the hypertrophic muscles.

To determine if nonmuscle myosin could be linked to the contractile response induced by PDBu we used blebbistatin, which is an inhibitor of actomyosin interaction. Blebbistatin targets nonmuscle myosin but has less effect on smooth muscle myosin (Limouze et al., 2004; Zhang et al., 2017). The specificity of blebbistatin has been discussed; the first study

(Limouze et al., 2004) reported a very low inhibitory effect on smooth muscle actomyosin, whereas a subsequent report suggested that it was equally potent on smooth muscle (Eddinger et al., 2007). A more recent report comparing blebbistatin effects on smooth and nonmuscle myosins, show however a clear selectivity toward nonmuscle myosin over smooth (Zhang et al., 2017), which is consistent with blebbistatin effects in organized smooth muscle containing nonmuscle myosin (Rhee et al., 2006; Ekman et al., 2005). We find that blebbistatin partially inhibits the high K^+ and carbachol induced contractions (Fig. 4 and 5 **Paper III**) consistent with a small inhibition of smooth muscle myosin. However, the PDBu induced contractions in the hypertrophied bladder muscle were inhibited by more than 85% by blebbistatin (Fig. 5 in **Paper III** and **Fig. 14** below).

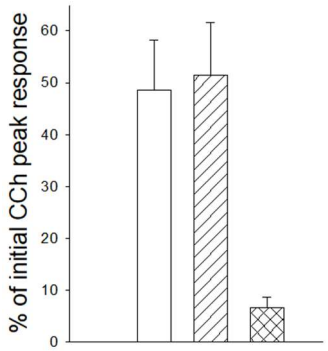

Figure 14. Contractile response to PDBu in hypertrophied urinary bladder smooth muscle. Direct activation of PKC with 100nM PDBu, in Krebs-Ringer solution: open bars (control), hatched bars (10 μM Y27632), crosshatched bars (10 μM blebbistatin, 10 μM Y27632). Figure modified from Fig. 5 in **Paper III**.

The main finding of **Paper III** is thus that hypertrophy of the urinary bladder in response to urinary outflow obstruction can lead to development of a structurally distinct wall component expressing nonmuscle myosin and activated by PKC. The physiological/pathophysiological mechanisms regulating this contractile component and if it can occur in the human bladder are unknown at present. Development of this structure might constitute a factor modulating the wall stiffness in bladder during hypertrophic growth.

4.8 ROTENONE REDUCES O_2 CONSUMPTION AND FORCE DEVELOPMENT

The aim of **Paper IV** was to examine the metabolic aspect of force development, based on the idea that the contractile process in smooth muscle would be dependent on the energy supply with a difference between fast and slow smooth muscle types, described in **Paper I**. We address this question by introducing a partial block of the respiratory chain (at complex I) in the mitochondria using rotenone (Swärd et al., 2002). As a first step, we verified that blocking complex I with rotenone affects the oxygen consumption of the tissue. In the relaxed smooth muscle (urinary bladder) rotenone inhibited the oxygen consumption by about 50%, giving a significant metabolic inhibition (right panel in **Fig. 15**).

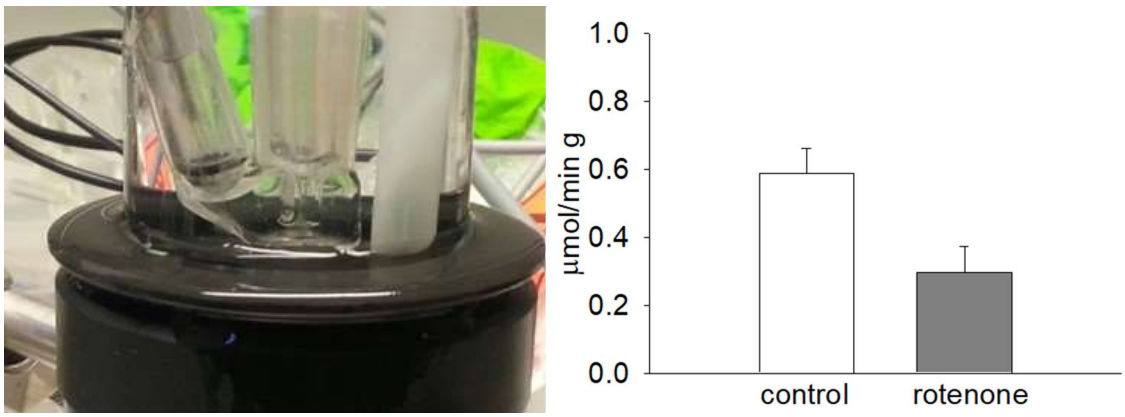

Figure 15. Oxygen consumption in relaxed smooth muscle. *Left panel:* Apparatus that holds muscle preparation in a closed glass chamber (37°C) equipped with a Clark electrode that was used to measure oxygen consumption during 10 min. *Right panel:* Oxygen consumption in relaxed smooth muscle from the urinary bladder; in control (DMSO) white bar and after inhibition with rotenone, grey bar. Figure based on data in **Paper IV**.

Active force of the smooth muscle was also significantly reduced by rotenone. Interestingly, the agonist induced active force in the rotenone treated tissue, was more affected in the fast (urinary bladder) compared to that of the slow (aorta) smooth muscle type, inhibited by about 50% and 30% respectively. The ATP turnover of the aorta is significantly lower than in fast muscle (Arner and Hellstrand, 1981) and it might thus be less affected by low energy supply. The low sensitivity to rotenone of the aorta is also consistent with the results from **Paper I**, where we observed that the aorta has a high expression of components in glucose uptake and glycolysis compared to the fast smooth muscle. It is thus possible that the aorta muscle better can support ATP generation via glycolysis and be metabolically more adapted and better prepared for an ischemic challenge. In this study we measured active force and it should be noted that other contractile parameters, mainly shortening velocity can be affected by ischemia/rotenone in a different way compared to force. It is expected that the shortening velocity slow aorta would be more sensitive to increased ADP levels (Löfgren et al., 2001).

Next, we tried to find a potential mechanism explaining the reduced force development in the rotenone treated smooth muscle tissue. We systematically examined potential cellular targets/pathways depicted in **Fig. 16** (below) using different pharmacological compounds targeting membrane channels (ATP activated and small and large conductance K^+ channels) and the metabolic sensor AMPkinase. We hypothesized that low ATP, induced by rotenone inhibition will lower ATP levels and increase ADP and AMP. A low ATP can activate K_{ATP} channels (Zünkler et al., 1988), hyperpolarize the membrane and lead to relaxation. Also, impaired Ca^{2+} removal via reduced activity of the sarcoplasmic reticulum pump (SERCA) can lead to Ca^{2+} activation of SK and BK K_{Ca} channels (Herrera and Nelson, 2002). If any such mechanism would apply, blocking of these K^+ channels should be able to reverse the rotenone effects on force. We report that neither of the applied channel blockers had this

effect (Fig. 2, **Paper IV**), excluding a major role of K^+ channel opening in the reduced force in metabolic inhibition.

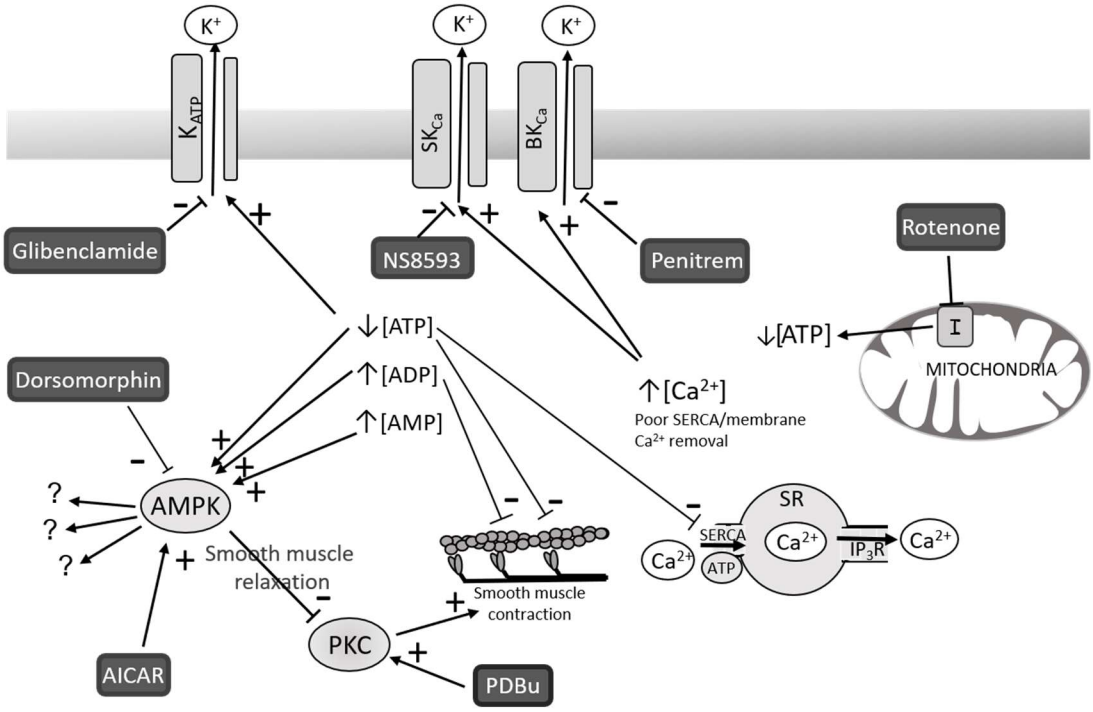

Figure 16. Potential cellular mechanisms affected by rotenone treatment. Simplified schematic of cellular mechanisms potentially underlying the reduced force in the rotenone treated tissue and the pharmacological compound used in **Paper IV** to target these mechanisms. Rotenone reduces oxygen consumption and ATP levels via effects on the mitochondria. Lowered ATP will potentially open K_{ATP} channels and affect Ca^{2+} removal with activation of K_{Ca} channels. Altered high energy phosphate levels will also affect the contractile system and activate AMPK.

The AMP-dependent kinase (AMPK) is a metabolic sensor activated by a change in metabolic status (Hardie, 2011). We have previously shown that activation of AMPK with AICAR leads to an inhibition of active force via an inhibition of protein kinase C and of the endothelial induced relaxation (Davis et al., 2012). It is possible that this mechanism is partially involved in the reduced force during rotenone treatment and in the impaired NO-mediated relaxation (Fig. 5, **Paper IV**). We however made the unexpected finding that inhibition of AMPK with dorsomorphin (an AMPK inhibitor, Pyla et al., 2014) dramatically potentiated the rotenone inhibitory effect (Fig. 4, **Paper IV**, and **Fig. 17** below). These results suggest that AMPK in smooth muscle (primarily the fast urinary bladder) has a protective action reducing the force inhibition during ischemia. The detailed mechanism remains to be explored, but in view of the effects of AMPK on glucose uptake (Musi and Goodyear, 2003; Nagata and Hirada, 2010) we suggest that AMPK is activated by the change in energy status, stimulating glucose uptake and promoting ATP generation via glycolytic pathways.

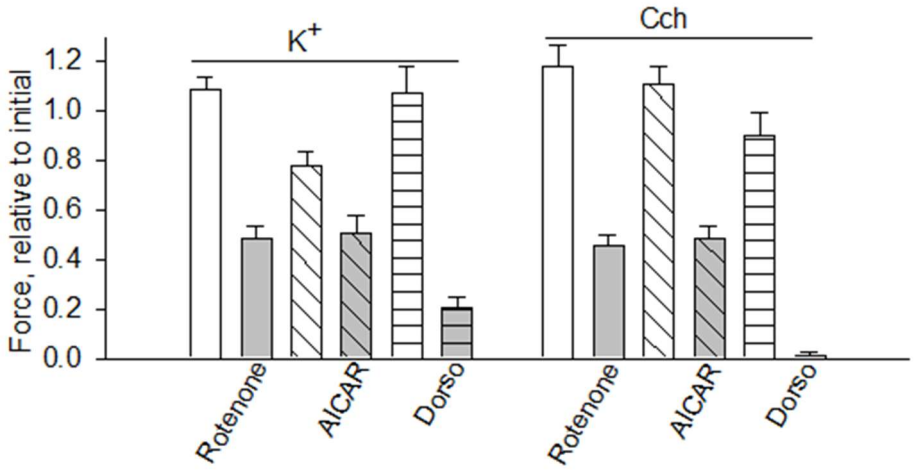

Figure 17. Relative inhibition in the presence of rotenone (filled bars) or with DMSO control (open bars) in the presence of AICAR (AMPKinase activator, diagonally hatched), or Dorsomorphin (Dorso, AMPKinase inhibitor, horizontally hatched) after activation with depolarization (KCl) or muscarinic agonist (carbachol, CCh) in the urinary bladdder. Figure based on data in **Paper IV**.

5 CONCLUSIONS

Paper I; A slow smooth muscle (versus a fast smooth muscle):

- found in large arteries
- slow cross-bridge turnover with low shortening velocity and high tension economy
- contractile proteins associated with slow actomyosin turnover
- slow kinetics in its deactivation (low phosphatase)
- high expression of components in Ca^{2+} sensitizing pathways and would thus be able to modulate its contraction via receptor activation
- energy consumption that is generally low
- ability to take up glucose, as well as lipid turnover is high, possibly related to the requirement of sustained energy supply and possibly also related to an increased sensitivity to extracellular glucose levels

Paper II; Hypertrophic growth induces changes in the urinary bladder:

- increased cholinergic responses, primarily due to post receptor changes
- lowered purinergic responses, due to alteration in nerve function
- increased Rho dependent Ca^{2+} sensitivity that correlated with higher RhoGDI and RhoA, and lower phosphatase (MYPT1)
- alterations correlates more with a slow smooth muscle, rather than a fast smooth muscle

Paper III; Hypertrophic urinary bladder can develop a nonmuscle myosin contractile component:

- mainly localized in the serosa
- activated by protein kinase C
- not a major part of the normal muscarinic contraction, but may contribute to wall stiffness and be activated by other (unknown) upstream pathways

Paper IV; Partial metabolic inhibition of mitochondrial complex I with rotenone:

- reduces the oxygen consumption to about 50%, thus induces a significant metabolic inhibition
- inhibits force development induced by depolarizing (high K+) and agonist induced contraction
- force development in fast smooth muscle (urinary bladder) is more sensitive to metabolic inhibition compared to the slow smooth muscle (aorta)
- AMPkinase has a significant protective action on the smooth muscle subjected to partial metabolic block

6 SVENSK SAMMANFATTNING

Glatt muskulatur är den typ av muskelvävnad som är icke viljestyrd och finns representerad i många olika organ och organsystem i kroppen, bland annat i blodkärl, urinblåsa, tarmarna, livmoder etc. Den är involverad i olika viktiga processer i kroppen såsom reglering av blodtrycket, tömning av urinblåsan, tarmarnas rörelse och livmoderns sammandragningar i samband med förlossning. Eftersom glatt muskulatur finns uttryckt på så många olika platser i kroppen är det inte förvånande att många olika sjukdomar och sjukdomstillstånd kan vara relaterade till förändringar i den glatt muskulaturen. Som exempel kan nämnas inkontinens vid förträngningar i urinvägarna, astma, och kärlförändringar vid diabetes.
Vi vet idag förhållandevis mycket om hur hjärt- och skelettmuskulatur fungerar och styrs, medan den glatta muskulaturens egenskaper är väsentligt mindre utforskade och klarlagda Studierna i denna avhandling innefattar försöksmodeller baserade på glatt muskelvävnad från försöksdjur (möss). I ett första arbete (**Arbete I**) visade vi att det finns olika typer av glatt muskulatur: snabba i tarm och urinvägar och långsamma i de stora kärlen. Dessa olika glatta muskeltyper är speciellt anpassade till sina unika funktioner i kroppen, tex upprätthålla blodtryck under låg energiomsättning i de stora kärlen eller dra samman urinblåsan för att tömma den. Snabba och långsamma glatta muskeltyper skiljer sig i kontraktila egenskaper, cellsignalering och metabolism. Vid olika sjukdomar och sjukliga tillstånd uppvisar glatt muskulatur en imponerande förmåga att anpassa sina egenskaper. Denna adaptationsförmåga studerades i det andra och tredje arbetet (**Arbete II och III**) i denna avhandling där vi visar att hypertrofisk tillväxt, liknande den som kan ske i urinblåsan vid t.ex. prostataförstoring, kan leda till förändrad cellsignalering med en ökad känslighet för kalcium som var medierad av en speciellt signalväg (Rho). Vi fann även att hypertrofisk tillväxt ledde till att det nervmedierade svaret förändrades, med en ökad känslighet för transmittorn acetylcholin vid direkt nervstimulering. Hypertrofisk tillväxt av urinblåsan kan leda till betydande uttryck av en speciell form av kontraktilt protein (non-muscle myosin, NMM), vilken är besläktat med de proteiner som utför kontraktionen i glatt muskulatur. Vanligtvis finns inte NMM i urinblåsa. Vi visar att hypertrofisk tillväxt av urinblåsan kan leda till utvecklandet av en unik kontraktil komponent, som kan aktiveras direkt via en unik signalväg (protein kinase C) och är beroende av NMM. Denna kontraktila komponent aktiveras inte vid normal kontraktion av urinblåsan, utan styrs av en separat (fortfarande okänd) mekanism. I ett sista arbete (**Arbete IV**) studerades hur kontraktionen i glatt muskulatur påverkas av en metabol blockering med rotenone. Rotenon ingriper i cellernas energiomsättning och hämmar syreförbrukning i mitokondrierna. AMPK är ett protein som känner av hur mycket energi som finns tillgängligt i cellen. Vi visar att metabol blockering minskar kraften i glatt muskulatur och den snabba muskeltypen är mycket mer känslig, jämfört med den långsamma glatta muskulaturen. AMPK kan delvis förhindra kraftminskningen vid metabol blockering med rotenone, och har sannolikt en skyddsfunktion vid energibrist och dålig blodförsörjning.

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"One picture is worth a thousand words", many thanks to Wordclouds.com for making it possible for me to summarize my whole thesis in a single picture (on the cover).

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